Die Dynamik von Assemblierungsprozessen des photosynthetischen Apparates der Grünalge *Chlamydomonas reinhardtii*

Doktorarbeit

vorgelegt von

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Introduction

1.) Photosynthesis

Photosynthesis is an energy converting process driven by light converting CO_2 into O_2 and sugar. In eukaryotic photosynthetic organisms this process happens in chloroplasts where light energy from the sun is converted into products useful for cell metabolism. By chemi-osmotic coupling energy derived from sunlight can be used to drive membrane-anchored protein pumps to generate an electrochemical gradient across the membrane. This H^+-gradient is necessary for the cell to drive energyconsuming reactions like ATP synthesis or metabolite transport. To reach this sun enenergy is absorbed by pigments of light harvesting complexes in the first step, which is then passed on to the reaction centres of the photosystems. The photosystems and other associated protein complexes make up an electron transport chain, where electrons are passed on from H_2O to CO_2 eventually. During this process O_2 and carbohydrates are formed, which are used up by cell respiration in mitochondria. The photons from sunlight can also be absorbed by Chl a-pigments of the reaction centres, but LHC proteins are indispensable for an efficient transduction of light energy.

1.1.) Primary Photosynthetic Reactions

The primary reactions of oxygenic photosynthesis occur on the thylakoid membrane and are catalysed by several protein complexes that consist of multiple subunits, pigments and redox cofactors. Besides about 100 proteins that are organised in the four major multisubunit protein complexes, namely photosystem I (PSI), photosystem II (PSII), the ATP-synthase complex and cytochrome b_6/f complex there are proteins that are engaged in assembly, regulation and maintenance of these proteins. The crystalline structures of PSI, PSII and *b_6/f* have been established at high resolutions (Ben-Shem et al., 2003; Jordan et al., 2001; Kurisu et al., 2003; Zouni et al., 2001) and gave detailed insights into the supra- and submolecular organisation of these protein complexes. Upon light absorption by the antennae, the excitation energy is channelled via pigments like chlorophylls and xanthophylls to the reaction centers to oxidise the chlorophyll dimers P680 and P700 in PSII and PSI, respectively. The electron donor side of PSII is capable of oxidising water to yield molecular oxygen and protons and electrons for the photosynthetic electron transport chain. The electrons originating from $P680^+$ are rapidly transferred across the thylakoid membrane to the final PSII acceptor quinones Q_A and Q_B. Once it is doubly reduced, Q_B diffuses into the plastoquinone pool and transfers its electrons to the cytochrome *b_6f* complex after binding to the Q_o site. In a series of complex reactions some of the electrons are transferred from the cytochrome *b_6f* complex via soluble plastocyanin to PSI while other electrons follow a different route within the cytochrome *b_6f* complex and participate in the proton pumping activity of this complex. Upon charge separation within PSI, the electrons are transferred from plastocyanin to ferredoxin and ultimately to NADP. (Nugent, 1996) Photosynthetic electron transfer generates a proton gradient across the thylakoid membrane that is used by the ATP synthase to produce ATP, which together with NADPH drives the Calvin cycle for CO_2 fixation. The thylakoid ATP synthase consists of nine

subunits that are organised in the transmembrane segment CF_0 and the hydrophilic segment CF_1 at the stromal surface. CF_0 translocates protons from the chloroplast lumen to the catalytic part of the enzyme and CF_1 catalyses the conversion of ADP and inorganic phosphate to ATP. (Yasuda et al., 2001)

1.2.) Assembly factors for photosystem I

The analysis of PSI mutants of *Chlamydomonas reinhardtii* has revealed a great number of cofactors involved in the assembly of this multi-subunit protein complex. Alone the production of the mature mRNA for the major subunit PsaA with 11 transmembrane spanning helices requires at least 14 nucleus-encoded factors (Goldschmidt-Clermont, 1998), whereas one factor is required for stability of PsaB mRNA and two for its translation. Synthesis of PsaA depends on ability to synthesise PsaB, while PsaB can assemble in the absence of PsaA synthesis (Stampacchia et al., 1997). The chloroplast genes ycf3 and ycf4 encode thylakoid membrane-located assembly factors for PSI and inactivation of one of the two cofactors in *Chlamydomonas reinhardtii* leads to a complete and selective loss of PSI (Boudreau et al., 1997). These genes have been found in all plastids examined and in cyanobacteria (Rochaix, 2002) and their non-expression has similar consequences in higher plants, though in cyanobacteria inactivation of ycf4 only leads to a partial loss of PSI (Ruf et al., 1997; Wilde et al., 1995). Ycf4 can be isolated by sucrose density ultracentrifugation as part of a high molecular weight complex (Boudreau et al., 1997).

2.) Structure and classification of LHC proteins

LHC proteins are also termed Chl a/b-binding proteins, which, in contrast to the photosystem subunits, reflects their ability to bind Chl b and other pigments like xanthophylls beside Chl a. Availability of chl seems to be crucial for the stable accumulation of chl-binding proteins (Falbel et al., 1996). LHC proteins span the thylakoid membrane with three α-helices. The threedimensional structure of the probably most abundant membrane protein in nature, the major LHCII-protein LHCb1, from pea, has been resolved by X-ray crystallography at a resolution of 3.4 Å which gave an insight into distribution and localisation of pigments inside the structure of this protein (Kuhlbrandt et al., 1994; Peterman et al., 1997). In LHCII lutein was identified as the main xanthophyll with two molecules per monomer. The two luteins occupy a central position of the protein as an internal cross-linker and probably contribute to the stability of the polypeptide and photoprotection (Formaggio et al., 2001). Other xanthophylls, especially neoxanthin were only found in substochiometric amounts and are probably peripherally bound to the complex.

The evolution to LHCs has probably led through two events of gene duplication from the High Light Inducible Proteins (HLIPs) of cyanobacteria which have only one transmembrane spanning helix (Montane and Kloppstech, 2000). This is documented in the only four-helix intermediate known today, which is PsbS. PsbS is an exceptional protein in this class for the C- and N- terminus are situated on the same side of the membrane (Funk et al., 1995). It is probably involved in nonphotochemical quenching and is a permanent constituent of the PSII reaction center (Li et al., 2000b). The three-membrane spanning helices proteins like ELIPs (Green et al., 1991), which accumulate under conditions of photooxidative stress (Montane et al., 1998), and LHCs have then further evolved through loss of one helix and are grouped into 10 different classes of nuclear encoded genes (Jansson, 1999). Lhca protein classes 1-6 constitute the LHCI complex associated

with PSI, Lhcb 3-6 are exclusively associated with PSII, while non-denaturing isoelectric focussing and immunoblotting showed that Lhcb1, Lhcb2 and Lhcb3 form mixed trimers (Jackowski et al., 2001).

A proteomic study of PSII antenna proteins from different plant species revealed that dicotyledones have a more diverse range of isoforms for Lhcb1, 3 and 6 than monocotyledones, with Lhcb1 showing the most isoforms. For Lhcb2, 4 and 5 in all species only one isoform was found (Huber et al., 2001). The diversity of Lhcb1 isoforms can not only be explained by posttranslational modifications but also by the existence of a family of closely related genes (Elrad and Grossman, 2004). Preparation of LHCI proteins on sucrose gradients results in two major subfractions. One is the Lhca1/4-fraction with a 77 K fluorescence emission maximum at 730 nm and the other is an Lhca2/3-fraction which at 77 K fluoresces maximally at 680 nm (Lam et al., 1984). Lhca1 and Lhca4 form heterodimers (Schmid et al., 1997), whereas Lhca2 and Lhca3 are not as tightly associated, but are in physical contact and require each other for stability (Ganeteg et al., 2001).

The gross architecture of LHC proteins is present for more than 350 million years, with a high degree of evolutionary conservation (Jansson and Gustafsson, 1991) and it is reckoned that all of these proteins have specific functions. In green plants and algae LHCs can make up to 25% of membrane proteins. The ten classes contain roughly 30 or more members with a high degree of organisation and interconnection (Jansson, 1999). There is a high copy number of more or less identical genes which are the templates for a high rate of protein synthesis. The diversity of expression products of light harvesting complex genes is increased by a number of posttranslational modifications. In a onedimensional separation of photosystem I from *Chlamydomonas reinhardtii* by SDS-PAGE seven distinct polypeptide bands could be assigned to Chl a/b-binding proteins (Bassi et al., 1992). But onedimensional protein separation is likely to fail in separation of

modification products because modifications like phosphorylation cause a shift in pH and not in size of the protein. Thus 2-dimensional protein separation using isoelectric focussing and SDS-PAGE is a more effective method in resolving all isoforms derived from expression of LHC genes (Hippler et al., 2001). But separation of membrane proteins has often proven to be a difficult task (Santoni et al., 2000b).

3.) Effect of Chl b-deficiency on assembly of LHCs

As before mentioned, each photosystem has it own set of light harvesting proteins, with some parts of them shared in cases of different demands for particular photosystems. On the other hand under different light intensities the accumulation of LHCs is adjusted to avoid under- or overexcitation of the photosystems (Anderson, 1986; Anderson and Osmond, 1987; Melis, 1992). Probably this is connected to synthesis and availability of the respective chl pigments.

There are a number of Chl b-deficient and Chl b-less mutants in a diverse range of algae and plant species. Chl b-deficient mutants generally show a lower overall chl-content, higher Chl a/b-ratios, smaller LHCII antenna population and reduction in size and number of grana stacks (Falbel et al., 1996; Falbel and Staehelin, 1994; Ghirardi and Melis, 1988; Greene et al., 1988a; Greene et al., 1988b; Harrison et al., 1993; Knoetzel and Simpson, 1991; Staehelin, 1986). Chloroplasts of the barley mutant chlorina f2 were found to have smaller photosynthetic unit sizes, decrease in LHCII and LHCI antenna size and a higher PSII/PSI ratio (Harrison and Melis, 1992), whereas the Chl b-less *Chlamydonas reinhardtii* mutant pg13 accumulates an unstable LHC-complex which is not detectable by lithium dodecyl sulfate polyacrylamide gel electrophoresis, though functionally equally effective (Michel, 1983). In contrast Arabidopsis plants overexpressing *cao*, a gene

responsible for Chl b-synthesis, increase the size of their photosystem II antenna (Tanaka et al., 2001). While it is clear that Chl b is not needed for the synthesis, transport, processing, or thylakoid insertion of the polypeptides in question (Bellemare et al., 1982), it was concluded that lack of Chl b leads to reorganisation of antennae and probably as a consequence of this reorganisation of PSI and II core complexes. Accumulation of Chl a- and Chl a/b-complexes is coupled to sequentiell synthesis of Chl a and Chl b respectively. In a situation of limited chl supply, chl is preferably integrated into apoproteins as Chl a. Chl b is derived from Chl a, but probably this reaction only occurs if excessive amounts of Chl a have accumulated, and reaction center proteins might have a higher affinity to the Chl a synthesising machinery. Besides Chl b is only found in antenna complexes. It is proposed that chlorophyll pigments are incorporatedc into monomeric LHCI and II apoproteins before their assembly into oligomers (Dreyfuss and Thornber, 1994a; Dreyfuss and Thornber, 1994b). Lack of Chl b integration might not prevent synthesis and membrane integration of LHC apoproteins, but Chl b-free LHC apoproteins are degraded in the thylakoid membrane, as found in the barley mutant f2 (Bellemare et al., 1982). The LHCI proteins Lhca1-3 and the LHCII protein Lhcb5 are stable in chlorina f2, whereas Lhcb2-4 are partially stable and Lhca4, Lhcb1 and Lhcb6 do not acumulate (Espineda et al., 1999; Harrison and Melis, 1992; Krol et al., 1995; Preiss and Thornber, 1995). To compensate for the loss of the predominant antenna protein Lhcb1, the PSII/PSI ratio is increased in the mutant to keep light harvesting as efficient as possible (Harrison et al., 1993). Chl b-deficient wheat mutants show a significant loss of Chl a/b-binding proteins (Falbel et al., 1996). Also thylakoid morphology is different in Chl b-deficient mutants. Compared to wildtype the amount of membrane stacking decreases in Chl b-deficient mutants in favor to long appressed thylakoid membranes. This phenotype becomes most severe under strong light irradiance (Falbel et al., 1996).

In *Chlamydomonas reinhardtii* the gene encoding the enzyme that catalyses the two dioxygenation steps involved in the production of Chl b from Chl a has been identified. The CAO gene encodes a chlorophyllide a oxygenase which contains putative binding domains for a [2Fe-2S]-Rieske center and for a mononuclear iron (Tanaka et al., 1998). *In vitro* assays with the recombinant enzyme from *Arabidopsis thaliana* showed that CAO converts chlorophyllide a into chlorophyllide b (Oster et al., 2000), although further enzymatic studies with CAO from *Arabidopsis thaliana* expressed in *E. coli* demonstrated that CAO requires no other protein factor to convert Chl a to Chl b (Rüdiger et al., 1999). Phylogenetic analysis of the chlorophyll a oxygenase genes from several prochlorophytes and chlorophytes showed that these genes share a common origin. This leads to the conclusion that the ancestor of chloroplasts had both Chl b and phycobilisomes (Tomitani et al., 1999). All oxygenic photosynthetic organisms contain Chl a, and are able to synthesise Chl b if they are transformed with CAO (Satoh et al., 2001). Expression of CAO and LHC genes is coordinated in acclimation of *Dunallelia salina* to irradiance stress (Masuda et al., 2003).

4.) Short-term adaptions to a changing environment

Light is an environmental factor of a fluctuating nature that exposes photosynthetically active organisms to short- and long-term changes. Whereas long-term changes are answered by developmental processes, transient short-term changes have to be adapted by physiological or biochemical cell processes to keep an internal steady-state. The photosynthetic apparatus is able to adapt to rapidly changing light conditions. In order to grow optimally oxygenic photosynthetic organisms need to optimise their photosynthetic yield under low light conditions and to dissipate

excess light excitation energy under high light to prevent oxidative damage. (Niyogi, 1999; Wollman, 2001)

In *C. reinhardtii* these mechanisms including state transitions and non-photochemical quenching have been successfully studied. Although some state transition mutants have been reported to show slightly increased values for nonphotocemical quenching, both processes are believed to be independently regulated and performed. (Elrad et al., 2002; Mussgnug, 2004)

4.1.) State transitions

PSII and PSI act in series to drive the photosynthetic machinery to produce reducing power for CO_2 fixation and ATP generation, but both photosystems have distinct preferences to certain light conditions. To avoid photooxidative damage and keep photosynthetic activity at an optimum, excitation levels of both photosystems have to be well balanced. Plants and green algae are able to react to changing light conditions by reversible distribution of excitation energy between the two photosystems in order to optimise quantum yield. This regulation mechanism is controlled by the redox state of the plastoquinone pool that triggers a signal transduction chain involving the $cytb_6/f$ complex and at least one LHCII-specific kinase. (Fig. 1) The enzyme activity of such a kinase has long been detected (Bennett, 1979) and LHCII kinases from Arabidopsis (Snyders and Kohorn, 1999; Snyders and Kohorn, 2001) and *Chlamydomonas reinhardtii* (Depege et al., 2003) have been identified. In Arabidopsis the PSII subunit PsaH seems to be necessary for excitation energy

transfer from LHCII to PSI under state II conditions (Lunde et al., 2000). In *Chlamydomonas reinhardtii* transition between the two states is accompanied by a larger decrease in fluorescence compared to Arabidopsis which makes the identification of defects and mutants easier (Wollman and Delepelaire, 1984).

While the cross-sections of LHC-complexes is almost equally balanced between the two photosystems in state I, about 80% of the LHCII antenna migrate to PSI in state II in *Chlamydomonas reinhardtii* (Delosme et al., 1996), whereas only 15 - 20% of the LHCII is mobile in higher plants (Allen, 1992). This fact can be exploited in mutant screens for state transitions-deficient mutants in *Chlamydomonas reinhardtii*, applying fluorescence video imaging (Fleischmann et al., 1999; Kruse et al., 1999). Loss of sensitivity against the linear electron flow inhibitor DCMU in state II and equal impairment of the state transition mutant stt7 in electron flow through $cytb_6f$ complex under state I and state II conditions together with the finding that plastoquinol binding in the Qo pocket of $cytb_6f$ complex is required for LHCII kinase activation indicate that transition to state II involves a switch from linear to cyclic electron flow (Finazzi et al., 2002; Zito et al., 1999). Thus for *Chlamydomonas reinhardtii* state transitions can be described as a way to produce ATP without further reduction of the plastoquinone pool, since depletion of intracellular ATP levels has also been found to induce state transitions in this organism (Bulte et al., 1990). According to LHCII kinases it is not clear if there is only one kinase that acts directly on LHCII or if the identified kinases are part of a signal transduction chain. Anyway, the kinases identified so far, TAK in Arabidopsis and stt7 in *C. reinhardtii* do not share major sequence similarities (Depege et al., 2003; Snyders and Kohorn, 2001). The potential membrane integration of stt7 is another indication that this kinase is actually a kinase kinase since the kinase directly responsible for LHCII phosphorylation is believed to be mobile. Also the targets of the kinases and the migrating part of LHCII have not been clearly identified.

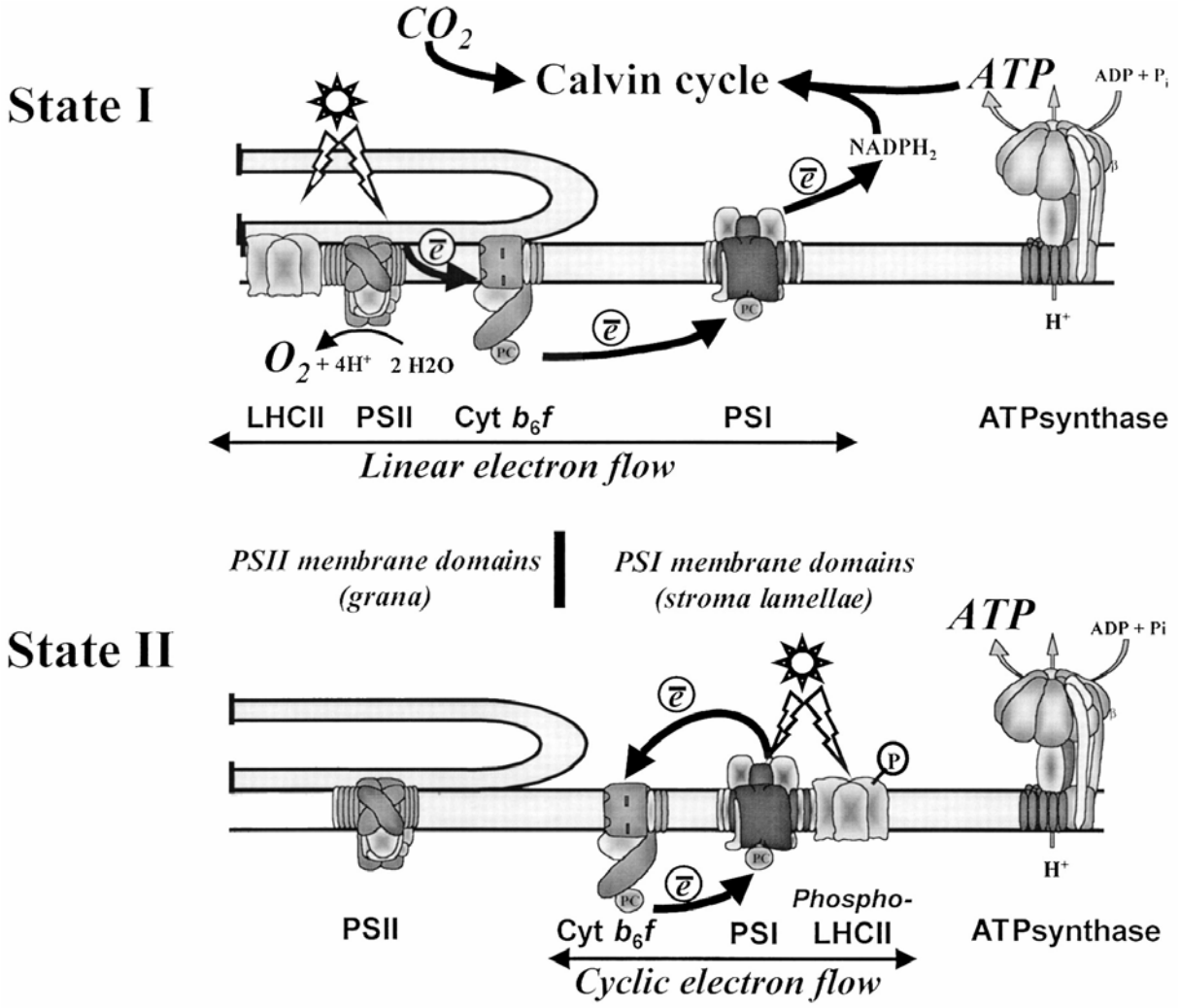


Fig. 1: The present view of state transitions: in state I, the supramolecular organisation of the photosynthetic apparatus is adapted to the fixation of CO_2 in the Calvin cycle. A linear electron flow from PSII (which extracts electrons from water leading to O_2 evolution) to PSI generates reducing power (NADPH) and ATP, both of which are required for the biosynthesis of carbohydrates. In state II, an extensive supramolecular reorganisation converts the photosynthesis apparatus in an ATP generator. A fraction of the major antenna proteins (LHCII) and cytochrome b_6f complexes undergo a lateral redistribution from the PSII membrane domains to the PSI membrane domains, which switches on a cyclic electron flow around PSI exclusively aimed at ATP synthesis. The flexibility in the functional organisation of the photosynthetic apparatus is established for work with the unicellular eukaryote *Chlamydomonas reinhardtii*. The extent to which it also applies to higher plant photosynthesis remains to be assessed (Wollman, 2001).

4.2.) Non-photochemical quenching

As an early measure of photoprotection thermal dissipation of excess excitation energy in LHCII decreases energy transfer to photosystem II (PSII) and reduces the formation of triplet chlorophyll in LHCII to prevent production of reactive oxygen species. Energy dissipation within LHCII or thermal dissipation results in nonphotochemical quenching of chlorophyll fluorescence (npq) that is reversed rapidly upon dissipation of the thylakoid membrane pH gradient (ΔpH) (Horton et al., 1996).

Nonphotochemical quenching is another photoprotective mechanism occuring in green algae and higher plants to minimise photooxidative damages by excess light. If photosynthetic organisms are exposed to increased light intensities, at a certain threshhold photosynthetic processes become saturated and absorption of excess light energy exceeds the capacity of photosynthetic complexes to utilise them. The chloroplast lumen then becomes highly acidic, the electron transport chain becomes reduced and excitation energy accumulates within the light-harvesting complexes of photosystem II (LHCII) leading to increased production of triplet chlorophyll a and singlet oxygen. Undetoxified singlet oxygen can cause protein modification and lipid peroxidation which can become autocatalytic and result in severe membrane destruction. Furthermore increased photosynthetic rates deplete the $NADP^+$ pool which causes an increase in the rate of electron flow from the donor side of photosystem I (PSI) to oxygen, generating superoxide and hydrogen peroxide, which can be scavenged by the water-water cycle under environmental stress conditions to dissipate excess photon energy (Asada, 2000). A short-term response to these excess light portions can be given by carotenoids that are able to detoxify and limit formation of singlet oxygen. The majority of the xanthophylls are bound to trimeric LHCII (LhcIIb) (Verhoeven et al., 1999).

Both the Lhcb trimers (Horton et al., 1991) and monomers (Bassi et al., 1993) have been hypothesised to be the actual sites of thermal dissipation in LHCII. The xanthophyll lutein, bound in LHC polypeptides can quench approximately 80% of the triplet chlorophyll generated within LHCII (Peterman et al., 1997). Singlet oxygen, generated by reaction of the remaining triplet chlorophyll with oxygen, can be detoxified by lutein and neoxanthin within LHCII or by zeaxanthin and α-tocopherol in the thylakoid membranes (Croce et al., 1999; Havaux et al., 2000).

Carotenoids have been shown to play a role in quenching chlorophyll triplet states (Siefermann-Harms, 1987), scavenge singlet oxygen (Croce et al., 1999) and ultimately down-regulate energy flow by dissipation of excess energy (Demmig-Adams, 1990). The kinetics of the $S_1 \rightarrow S_n$ transition of xanthophylls (530-580 nm) were found to be significantly different under maximum and no npq. At maximum npq this difference seems to correlate with the accumulation of zeaxanthin (Ma et al., 2003).

Components of the α-branch and the β-branch of carotenoid biosynthesis are important for nonradiative dissipation of excess absorbed light energy (Niyogi et al., 1997). Zeaxanthin, antheraxanthin and lutein have ten or more double bonds and at least one cyclohexenyl ring with a single oxygen substituent making direct transfer of excitation energy from ^{1}chl to zeaxanthin, antheraxanthin and lutein energetically more feasible than to violaxanthin and neoxanthin which have fewer than 10 conjugated double bonds. Lutein, violaxanthin and zeaxanthin are crucial in xanthophyll-dependent npq, with zeaxanthin showing maximal fluorescence quenching capacity in its LHCII-bound state, which might as well be related to photochemical properties as well as conformational changes of the protein environment induced by binding of the specific carotenoid

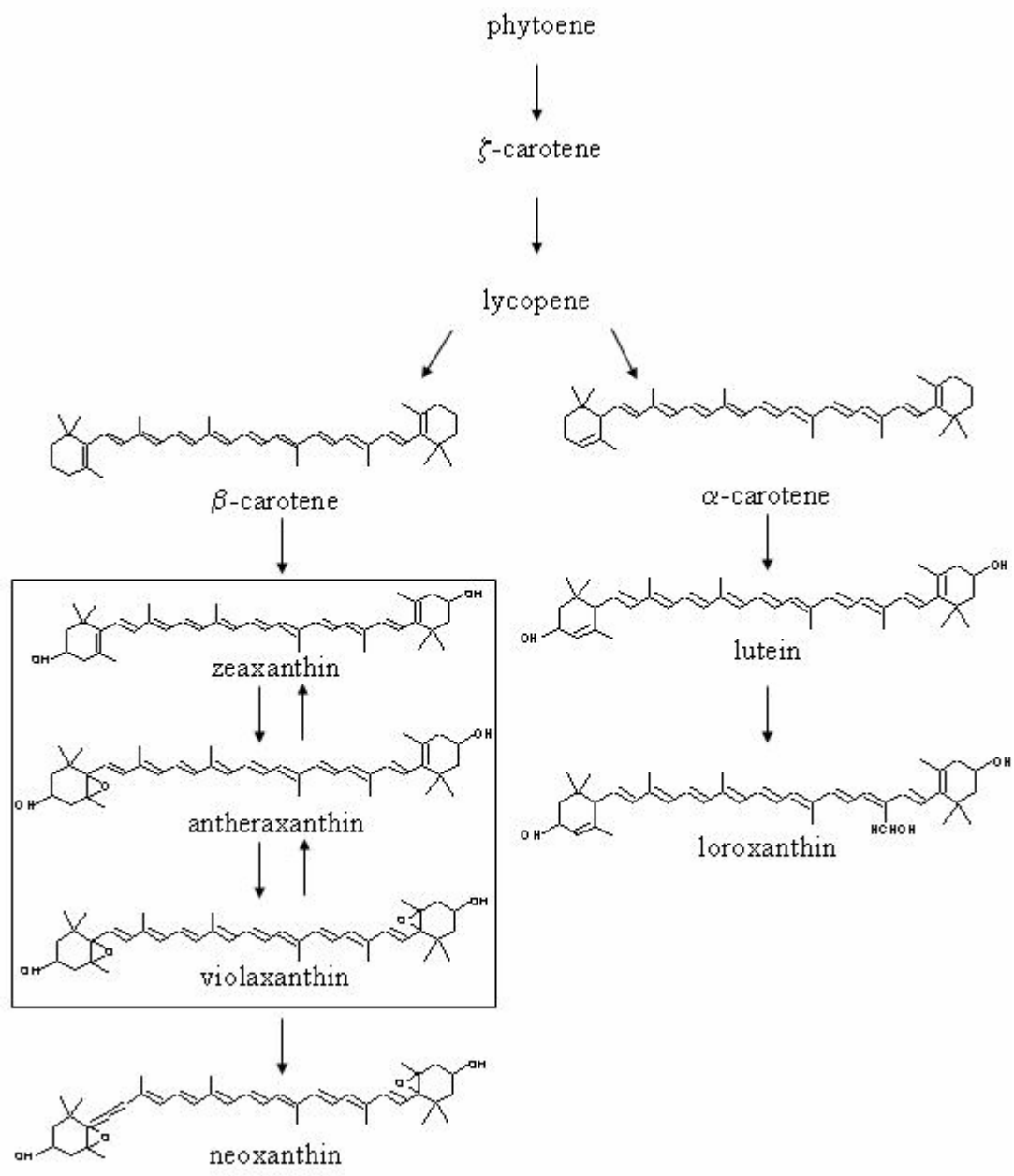


Fig.2: Biosynthetic pathway for β-carotene branch and α-carotene branch carotenoids. The boxed region indicates thje steps involved in the xanthophyll cycle. At normal light conditions and high lumen pH zeaxanthin epoxidase is active which ultimately leads to synthesis of violaxanthin and neoxanthin. High light conditions and overexciatation of pigment molecules ultimately lead to activation of the thylakoid lumen. At low lumen pH violaxanthin deepoxidase is activated which leads to the formation of zeaxanthin.

(Polivka et al., 2002). At low lumen pH violaxanthin deepoxidase is activated which leads to the formation of antheraxanthin and ultimately zeaxanthin from violaxanthin. (Fig. 2) The phenotypes of Arabidopsis mutants that lack either a functional violaxanthin deepoxidase or PsbS, which is essentiell for npq (Li et al., 2000b) and double mutants exhibiting both deletions, strengthened the

view that enhanced protection against photooxidative damage and antioxidant activity by the formation of zeaxanthin through the violaxanthin cycle is only partially related to quenching of ^{1}Chl in the LHCs (Havaux and Niyogi, 1999).

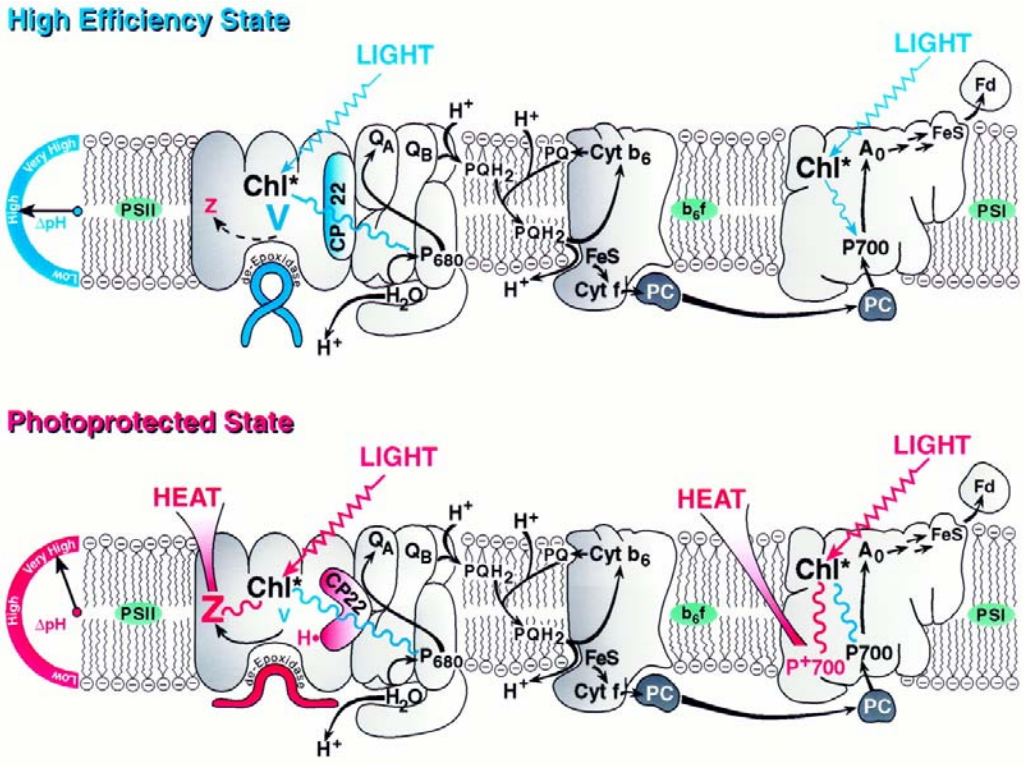


Fig. 3: Model depicting events induced by excess light that lead to the conversion of the thylakoid membrane from the high efficiency state (top) to the photoprotected state (bottom) (Ort, 2001). The excess light condition is sensed by a very large **Δ**pH that initiates the non-photosynthetic thermal dissipation of absorbed light as described in the text. The major elements involved in the conversion between the high efficiency and photoprotected states are highlighted by the transition from blue to red. Main events are overexcitation of chlorophyll molecules, dissipation of light energy as heat or quenching by carotenoids, protonation of PsbS (CP22), activation of violaxanthin deepoxidase and production of zeaxanthin from violaxanthin. (Ort, 2001)

To elucidate aspects of npq *Chlamydomonas reinhardtii* mutants have been created by insertional mutagenesis and npq-defected mutants were identified by video imaging of fluorescence. The mutant npq5, not being obviously impaired in photosynthesis or xanthophyll cycle, proved a rewarding tool to elucidate the role of a specific LHCII in thermal dissipation (Elrad et al., 2002). Molecular analysis of the mutant which was created by insertional mutagenesis revealed that the

Lhcbm1 gene was deleted, and complementation of the strain with a Lhcbm1 genomic clone restored the decreased npq values. The reduction in LHCII trimers in this strain was reflected in an equivalent reduction in pigments including the xanthophylls neoxanthin and loroxanthin and chlorophyll b. Since ΔpH is large enough to elicit thermal dissipation, expressed in the proper functioning of the xanthophyll cycle, and the reduction in npq is unproportional to the recuction in LHCII trimers, it seems likely that Lhcbm1 serves a specific function in npq. So far, the main factors identified to play a role in npq are ΔpH, which is caused by excess excitation energy, proton binding to PsbS and other PSII proteins, LHCII as the binding site for carotenoids and activation of the xanthophyll-cycle that leads to the formation of the particularly photoprotective and antioxidative xanthophyll zeaxanthin. (Fig. 3)

5.) Adaption to Fe-deficiency

Iron is a crucial element in the metabolism of all organisms, since Fe is incorporated in heme and iron-sulfur clusters in a variety of key metabolism enzymes like hemoglobine, the cytric acid cycle enzyme aconitase, the nitrogenase complex and enzymes involved in the mitochondrial oxidative phosphorylation chain or the photosynthetic machinery. Most of these enzymes play an important role in electron transfer, but some also have a sensory function for oxidation states and control gene expression (Beinert and Kiley, 1999). Therefore a deficiency in iron supply causes a serious nutritional problem to all procaryotic and eukaryotic forms of life. Among them photosynthetic organisms are most severely affected by iron-deficiency since the photosynthetic apparatus has a particular demand for Fe. The typical phenotype of Fe-deficiency in plants, green algae and cyanobacteria is marked by chlorosis which goes hand in hand with reduction of the photosynthetic machinery (Spiller and Terry, 1980). Photosystem I complex which is a very abundant protein in

photosynthetic membranes contains 12 Fe molecules which are bound in three Fe_4S_4-clusters F_x, F_a and F_b. Specific and early loss of PSI centers which is the major iron sink in the chloroplast in conditions of iron-deficiency is a hint that Fe can be mobilised from PSI. In cyanobacteria the PSI:PSII ratio is reduced from 4:1 to 1:1 under iron-stress (Straus, 1994), while induction of the iron-stress induced gene isiA leads to accumulation of a protein that forms a ring of 18 molecules, each containing 12 chlorophylls, around PSI trimeric reaction centers (Bibby et al., 2001; Boekema et al., 2001; Laudenbach and Straus, 1988). The function of this shield of chlorophylls could be light harvesting in substitution for degraded phycobilisomes or to dissipate excess light energy (Michel and Pistorius, 2004; Park et al., 1999; Sandstrom et al., 2002; Yousef et al., 2003).

In *Chlamydomonas reinhardtii* it was found that under Fe-deficiency besides partial degradation of both photosystems and cyt b_6/f complex levels, the amount of LHCI antennae were drastically reduced, whereas levels of LHCII proteins stayed fairly constant (Moseley et al., 2002a). The degradation and remodeling of LHCI proteins as an adaptation progress to overcome light intensity caused by loss of PSI turned out to be a sequentiell progress. As a first consequence antenna proteins are uncoupled from PSI, followed by degradation of specific LHCs and induction of new LHCs, eventually leading to a reshape of the antenna complexes. Thereby PsaK has been suggested as a factor responsible for uncoupling of antenna proteins. (Moseley et al., 2002a) Accumulation of PsaK itself might be influenced by the iron enzyme Crd1, which has been suggested to be affected by regulation mechanisms following Fe-deficiency. Crd1 encodes a protein with a di-iron motif with a similarity to a protein implicated in the cyclase reaction in the chlorophyll biosynthetic pathway. (Moseley et al., 2000; Moseley et al., 2002b) The Crd1 mutant of *Chlamydomonas reinhardtii* shows a similar phenotype of chlorosis in copper-depleted medium as does the wildtype under Fe-deficiency, indicating that there is an interconnection of iron and copper metabolism (Moseley et al., 2000; Moseley et al., 2002a). Like iron copper is an essential transition metal and

an important cofactor in enzymes and electron transfer proteins like cytochrom oxidase and plastocyanin that are involved in oxygen chemistry or redox reactions. Algae and cyanobacteria are able to replace copper-dependent proteins by alternative iron-dependent proteins under Cu deficiency, thus plastocyanin can be replaced by the heme protein cyt c_6 (Wood, 1978). The ferric reductase which reduces Fe(III)-chelates to transportable Fe(II) is also capable of reducing exogenous Cu(II)-chelate, and has been shown to be stimulated by either Fe-deficiency or Cu-depletion of pea seedlings (Welch et al., 1993). Induction of ferric reductase by Fe deficiency led to a significant increase of copper concentrations in leaves of pea seedlings. (Evans et al., 1992)

6.) Proteomics

For *Arabidopsis thaliana* estimations range from about 2000 - 5000 proteins that are targeted to the chloroplast, whereas a number of 2400 proteins, encoded by nucleus or plastid, seems most realistic (Abdallah et al., 2000; Martin and Herrmann, 1998). Proteomics, that means analyses of whole enclosed units of metabolism from whole organisms to cellular subcompartiments on a protein level, has become a common tool to reveal sequences, modifications and functions of proteins expressed at a certain time and place. The standard techniques for such undertakings are normally 2-DE including isoelectric focussing and SDS-PAGE and subsequent analyses of tryptic digests by mass spectrometry. No twodimensional map of intrinsic thylakoid membrane proteins has been established prior to the approaches presented in this dissertation and (Hippler et al., 2001; Stauber et al., 2003). However, from a twodimensional dissection of soluble lumenal and peripheral thylakoid proteins of pea it has been estimated that there are from 200 - 230 proteins in this category. It can be postulated that the thylakoid membranes contain a large number of other proteins that are involved in the biogenesis and regulation of the four multisubunit complexes (Wollman et al., 1999). These

additional proteins would be involved in processes such as biosynthesis and ligation of cofactors, insertion of proteins into membranes, and folding and degradation of proteins.

6.1.) Separation of membrane proteins

From preparations and extracts of biological matter membrane proteins have always been found difficult to isolate. The poor resolution of membrane proteins has several reasons, among them their embedment in lipid components, high hydrophobicity and often their high alkalinity. This is on the one hand due to their rather low abundance and on the other hand caused by their varying degree of membrane association and hydrophobicity which makes them less liable to go into solution than cytosolic proteins. The requirement for specific extraction and solubilisation methods if membrane protein identification has to be included in the output of the analysis has created a wide range of methods and tools for the proteome analysis of membrane proteins. Preparation of a bilayer protein matrix for mass spectrometric analysis requires a change to the protocols that are otherwise used for the preparation of cytosolic proteins.

6.1.1.) Isolation of membranes

Although failure to detect hydrophobic proteins is often blamed on their low abundance and restraint to go into solution, it is obvious that reduction of sample complexity by adequate preparation and fractionation of membrane matrices is a crucial step towards a comprehensive analysis of its components (Molloy et al., 1999). For example, direct trichloroacetic acid precipitation of pelleted whole cell suspensions can reveal membrane proteins, but the majority of

the more hydrophobic ones might be missed (Eschenbrenner et al., 2002). One of the most frequently applied methods in that context is density gradient ultracentrifugation. Here the fact is used that different membrane species have a different flotation behaviour in a percoll or sucrose solution of a certain molarity than other membrane types or cell components. There are specified protocols for isolating membrane species of different subcompartments by sucrose gradient ultracentrifugation (Hoppel et al., 2002; Maltman et al., 2002)

6.1.2.) Precipitation and enrichment

The first step in obtaining membrane proteins for analysis is to take them from the grip of their lipid bilayer environment. The proteins have to be recovered from the membranes by precipitation under hydrophobic conditions. Chloroform-methanol precipitation is a rather effective method to recover membrane proteins without discriminating the soluble protein components of the sample (Hippler et al., 2001; le Coutre et al., 2000; Wessel and Flugge, 1984), but other organic-assisted extraction methods using trichloroacetic acid (Havlasova et al., 2002), methanol (Blonder et al., 2002), acetone (Cullen et al., 2003) or both of the latter mentioned in combination with tributylphosphine (Mastro and Hall, 1999) also proved helpful.

6.1.3.) Solubilisation

Once the protein is recovered from the lipids it is necessary to use chaotropic nonionic or zwitterionic detergents like CHAPS or thiourea for solubilisation of membrane proteins prior to IEF (Rabilloud et al., 1997). But apparently there is not the one master recipe for solubilising membrane

proteins, although highly chaotropic reagents including thiourea seem to be irreplaceable if membrane proteins have to be solubilised (Molloy et al., 1999; Pasquali et al., 1997; Rabilloud et al., 1997) and is consistently used in most applications. But the appropriate composition of solubilising agents has to be screened for, often by trial and error, to find the ideal conditions for a specific organism, cell type or organell (Carboni et al., 2002; Cullen et al., 2003; Pessione et al., 2003; Phadke et al., 2001).

6.2.) 2-D-PAGE

Since its invention in 1975 (O'Farrell, 1975) high resolution twodimensional electrophoresis using subsequent isoelectric focussing and SDS-PAGE of protein samples has been the method of choice in establishing protein maps. Not only can protein maps of different samples be compared, 2-DE additionally offers the possibility to isolate components of a complex protein sample into defined spots, ready for individual picking and analysis. Thus, 2-DE allows a more thorough mass spectrometry of individual spots, in contrast to shotgun proteomics approaches like MudPIT (multidimensional protein identification technology) where reduction of sample complexity only takes place at the stage of peptide separation by liquid chromatography, after a mixture of different membrane proteins has been tryptically digested, often from a whole tissue or cell extract (Gomez et al., 2002; Kislinger et al., 2003; Koller et al., 2002; Wolters et al., 2001). Liquid chromatography in combination with mass spectrometry and database searching can offer a powerful tool in random analysis of complete proteome digests without the need for 2-DE (Washburn et al., 2001). Although such approaches are often more rapid and inexpensive, and more comprehensive on a proteome level, protein sequences can often not be as comprehensively and accurately covered since individual protein identification must be done from fewer peptides. Furthermore reports claim that

extensively membrane-spanning proteins can not be detected from liquid snapshots due to high hydrophobicity (Kolker et al., 2003). A recent shotgun approach in lung and liver tissues from mice resulted in reliable identification of over 2.000 proteins from 300.000 mass spectra, among them more than 100 integral membrane proteins, allowing conclusions to their subcompartiment localisation and function (Kislinger et al., 2003). Such an example demonstrates the advantages in rationality that shotgun approaches can provide compared to 2-DE approaches, but to address specific questions more directly targeted approaches might be useful.

But although proteins have been detected with high resolution and reproducibility, membrane proteins have often been shown to be underrepresented on 2-DE gels in relation to their intracellular quantity, partly due to their poor solubilisation behaviour (Bauer et al., 2001; Bruyns et al., 1998; Lubec et al., 2003; Santoni et al., 2000a), so in some cases alternative strategies had to be applied. Thus, besides adequate fractionation and precipitation of membranes as mentioned above, an effective solubilisation procedure is essentiell for satisfactory resolution of membrane proteins.

6.3.) 1-D-PAGE

But expensive and time-enduring 2-DE is not the only choice of separating proteins for further analysis. Concerning solubilisation 1-dimensional SDS-PAGE has the advantage over 2-DE that conditions are not so demanding. Membrane proteins are well separated in 1-dimensional gels, even under non-denaturing conditions. An advantage of 2-D-gels certainly is that you can pick single spots that contain only a few different proteins if not only one protein species. This reduces the diversity of the peptide mixture and makes MS analysis easier. Furthermore a quantitative comparison of single protein spots, e.g. if isolated from different conditions can be achieved. But

with the increasing raffinity of pre-MS and MS preparation and analysis tryptic digests from 1-D-bands can also be handled nowadays with low detection limits and high accuracy (Li et al., 2000a).

Blue Native PAGE is a method which is often employed in the separation of mitochondrial membrane complexes (Schagger, 2001; van Lis et al., 2003). It offers a mild but efficient separation method which allows separation of protein complexes in their native state, less discriminating for basic pI-values than IEF. 2-dimensional electrophoresis as a sequence of 1-dimensional PAGEs, with a native PAGE as a first dimension can help to improve the resolution of membrane complexes (van Lis et al., 2003). Such an approach also proved effective in identifying thylakoid membrane components (Rexroth et al., 2003). Different prospects and procedures using blue native gel electrophoresis as a method to study mitochondrial protein complexes and patterns are described in (Nijtmans et al., 2002).

Another native protein separation method, Deriphat-PAGE helped as a first dimension to resolve and identify thylakoid membrane proteins in a twodimensional pattern (Peter and Thornber, 1991).

6.4.) MS

Early membrane proteome analyses have been limited to comparing 1-dimensional protein patterns separated by SDS-PAGE (Fujiki et al., 1982) or autoradiography (Chaney and Jacobson, 1983), but with increasingly refined mass spectrometry techniques to analyse protein sequences, a lot of membrane proteins have been microsequenced during the last decade.

Powerful mass spectrometry tools have been developed for the analysis of tryptic digests from 1-D and 2-D gels (Hunter et al., 2001; Shevchenko et al., 1996), some specialised on the detection of membrane proteins (Li et al., 2000a; Pedersen et al., 2003)

7.) Membrane proteomics approaches in plants and algae

In plants many projects have focussed on the membrane content of energy-transducing organella, but also matrices like the plasma membrane or the peribacteroid membrane in species bearing symbiontic bacteria have been closely examined. To date Arabidopsis and rice are the two higher plants genomes which are completely sequenced, while proteome studies in *Chlamydomonas reinhardtii* also have a strong support from profound genomic databases.

In an attempt to analyse the proteome of the suppressed thylakoid membrane subdomains in *Pisum sativum, Spinacia oleracea* and *Nicotiana tabacum*, membrane preparations were enriched for PSII-subunits by Triton-treatment. Reverse-phase chromatography coupled to electrospray ionisation mass spectrometry allowed the detection of several intact hydrophobic membrane proteins. Posttranslational modifications could be identified, although distinct phosphorylation sites could not be attributed (Gomez et al., 2002). Intact photosystem II center proteins from *Spinacea oleracea* were measured by ESI-MS with high mass accuracy and the detergent-isolated core complex of PSII has been characterised by ESI-MS and fast atom bombardment (FAB)-MS allowing conclusions about their subunits and pigment composition and modification states (Sharma et al., 1997a; Sharma et al., 1997b; Sharma et al., 1997c; Whitelegge et al., 1998; Zheleva et al., 1998). Analysis of a his-tag-purified PSII complex revealed a range of novel subunits in the cyanobacterium *Synechocystis sp. PCC 6803* (Kashino et al., 2002). Improvements in liquid

chromatography separation of proteins and peptides have improved detection limits and accuracy of mass spectra, examplified in the characterisation of photosystem II antenna complexes of higher plants with ESI-MS (Premstaller et al., 2001). Whereas for the analysis of pea and spinach *Arabidopsis thaliana* genome data had to be used, the complete sequencing of the *Chlamydomonas reinhardtii* genome is helpful for proteomics in this unicellular green alga. Separation of digitonin-solubilised thylakoid membranes by Blue Native PAGE and subsequent Tricin-SDS-PAGE allowed resolution of subunits of photosynthetic reaction centers, light harvesting complexes and other thylakoid membrane complexes. Thus in combination with in-gel-digestion and MALDI-MS it was possible to identify the subunits of the photosynthetic machinery of *Chlamydomonas reinhardtii* (Rexroth et al., 2003). Onedimensional SDS-PAGE of chloroform-methanol extracted proteins was the separation method of choice in an effort to anlyse the hydrophobic subproteome of the chloroplast envelope in *Spinacia oleracea*. In comparison with an *in silico* approach to identify transmembrane-spanning envelope transporter proteins, the proteomic data showed that bioinformatic data retrieved from the *Arabidopsis thaliana* genome database would fail to predict the chloroplast membrane location of many transporter systems. (Ferro et al., 2002) An estimation of the beta-barrel proteome of the chloroplast outer envelope membrane was undertaken in a combined effort of *in silico* and proteomic tools, basing on its evolutionary relation to the outer membrane of gramnegative bacteria. Four new proteins of the outer envelope membranes of *Pisum sativum* were identified. (Schleiff et al., 2003) A differential proteomics approach in chloroplasts of an *Arabidopsis thaliana* envelope transporter mutant using 2-dimensional difference gel electrophoresis (DIGE) and mass spectrometry helped to elucidate the import of nucleus-encoded proteins into chloroplasts (Kubis et al., 2003).

By applying Blue Native PAGE and N-terminal sequencing for mitochondria of *Chlamydomonas reinhardtii*, major mitochondrial respiratory complexes were visualised and identified by

immunoblotting or N-terminal degradation (van Lis et al., 2003). By subfractionation of mitochondrial proteins integral membrane proteins, although not the most hydrophobic ones, could be identified by peptide mass fingerprinting from 2-dimensional gels (Millar et al., 2001). The before mentioned procedure was modified through Na_2CO_3 treatment of isolated membranes and a more sophisticated MS approach, enabling to identify tryptic digests obtained from 1-dimensional SDS-PAGE bands. Focussing on a transmembrane carrier family in mitochondria of *Arabidopsis thaliana* six of the nine carriers were identified that have not been resolved in the before mentioned 2-DE analysis due to their high GRAVY scores. The most hydrophobic peptide identified with a hydrophobicity value of 3.66 indicates the hydrophobicity limit for extraction and ionisation. (Herald et al., 2003)

Identification of ER proteins of the developing oilseed in Arabidopsis was enabled by separating the proteins into fractions of different hydrophobicity by gradient centrifugation and analysing them by 1- and 2-dimensional gel electrophoresis and mass spectrometry (Maltman et al., 2002).

By phase partitioning with Triton-X integral and peripheral fractions of membrane proteins of the plasma membrane of *Arabidopsis thaliana* and other organelles can be obtained and resolved by 2-DE. While early studies revealed mainly peripheral membrane proteins (Santoni et al., 1998) due to improvement of solubilisation procedures and the establishment of organelle marker databases (Prime et al., 2000; Santoni et al., 2000b), later studies were successful in identifying integral membrane proteins, although with some contamination from intracellular membranes (Kawamura and Uemura, 2003; Santoni et al., 2003). Aquaporins are a well-studied and abundant constituent of leaf plasma membranes, a number of them could be identified by proteomics, giving insight into differential expression (Fotiadis et al., 2001) and phosphoregulation (Santoni et al., 2003).

The symbiosome is a specific compartment in legumes associated with symbiosis with nitrogen-fixing bacteria. This compartment harbours the bacteroids surrounded by a peribacteroid membrane (PBM) originating from the plant plasma membrane. Whereas in one study only PBM-associated proteins were identified (Panter et al., 2000), other authors found a number of endomembrane proteins in the PBM fraction which fed theories of the endomembrane being involved in PBM biogenesis (Saalbach et al., 2002; Wienkoop and Saalbach, 2003).

In this dissertation an approach using 2-DE in combination with immunoblotting, liquid chromatography and mass spectrometry to dissect the proteome of thylakoid membranes in *Chlamydomonas reinhardtii* is discussed, which resulted in a proteome map of the light harvesting complexes, showing the modification pattern of this family of proteins and giving clues to possible functions and regulations (Hippler et al., 2001; Stauber et al., 2003).

8.) Tracking the phosphorylation status

Different techniques have been applied in the study of phosphorylation like isoelectric focussing, radiolabeling, Edman-degradation, enrichment of phosphopeptides and different MS-approaches.

Whereas experiments performed in the 70's using isoelectric focussing (Kuhn and McDowell, 1977) or radiolabeling (McDowell and Kuhn, 1977) could only tell about the quantity or conditions that induce phosphorylation, later experiments based on mass spectrometry could give conclusions about the actual phosphorylation sites of a protein. It was soon found out that mass spectrometry has an advantage over N-terminal sequencing and other sequencing techniques, especially when it comes to defining sites of modification. The MS techniques applied in identifying phosphorylation

sites comprise MS-MS (Biemann and Scoble, 1987), fast-atom bombardment mass spectrometry (Holmes et al., 1987; Poulter et al., 1987), Fourier-transform mass spectrometry (Stensballe et al., 2000), plasma desorption mass spectrometry (Tsarbopoulos, 1989), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI) (Ruotolo et al., 2002; Yip and Hutchens, 1992; Zhang et al., 1994) and electrospray ionisation mass spectrometry (Affolter et al., 1994; Wilm et al., 1996; Zolodz and Wood, 2003) or combinations of them (Kouach et al., 1994). Detection of fragmentation loss of specific marker ions like HPO_3 (80 Da) and H_3PO_4 (98 Da) is often essentiell for identifying monophosphorylated peptides (Beck et al., 2001). Before applying mass spectrometry it can be helpful to get an overview of the phosphorylation pattern by immunoblotting with antiphosphoserin, -tyrosine or -threonine-anibodies (Yanagida et al., 2000) or by fluorescent-dye labeling of 1-DE or 2-DE separated phosphoproteins (Steinberg et al., 2003). Also prepurification of phosphopeptide mixtures by metal-ion-affinity-chromatography before application to mass spectrometry proved a valuable tool (Neville et al., 1997; Posewitz and Tempst, 1999; Schlosser et al., 2002; Stensballe et al., 2000). Affinity chromatography based on immunoprecipitation of phosphoproteins using anti-phosphoserine or -threonine antibodies is also a valid method (Mann et al., 2002). Different procedures of phosphoserine- and phosphothreonine-specific β-elimination and other techniques for chemical modification make phosphopeptides perform more detectable over untreated peptides in mass spectrometry (Adamczyk et al., 2001; Zhou et al., 2001; Zhu et al., 2002). Digestion of peptides modified by phosphorylation can be performed more effectively with more specific endopeptidases (Knight et al., 2003). Chemically modified phosphomoieties can be specifically selected and allow quantitation between different samples, especially if they are isotopically or / and biotin- or avidin- labeled (Adamczyk et al., 2002; Bonenfant et al., 2003; Goshe et al., 2002). Also labeling with fluorophores has been introduced as a quantitation method (Fadden and Haystead, 1995). Quantification is also possible by applying inductively coupled plasma mass spectrometry (ICP-MS) (Wind et al., 2001). The

native reference peptide method allows stoichiometric quantitation of phosphorylation states during a physiological time course (Ruse et al., 2002).

In thylakoids phosphorylation plays a role in the life cycle of the PSII reaction center protein D1 and the phosphoregulation of LHC proteins in light adaptation processes. Phosphorylation of LHCII and PSII proteins has been successfully identified and localised (Fleischmann et al., 1999). CNBr cleavage proved a more specific tool to sequence the PSII reaction center proteins D1 and D2, purified by electroelution. A range of MS-techniques was applied to reveal the modification sites of the proteins (Sharma et al., 1997c). Intact mass measurements of undigested polypeptides from isolated spinach-PSII-complexes were performed with ESI-MS and phosphorylation sites in a number of PSII core and antenna subunits could be identified. This was possible by identifying mass adducts of +80 Da. Mass adducts of +32 Da were ascribed to dioxygenation, probably associated with a higher degree of phosphorylation and photodamage (Gomez et al., 2002). Treatment of thylakoid membranes from *Arabidopsis thaliana* with trypsin and enrichment of the cleaved extramembraneous phosphopeptides by metal affinity chromatography enabled identification of phosphopeptides that do not belong to the PS II supercomplex for the first time (Hansson and Vener, 2003). Digesting thylakoid membranes from spinach with proteinase K or thermolysin proved effective in releasing phosphopetides that could be enriched by ferric ion affinity chromatography and reverse phase HPLC and identified by fragmentation loss of 98 Da phosphoric acid. These phosphopeptides could be assigned to light harvesting proteins (Michel et al., 1991). From a 2-DE pattern of *Arabidopsios thaliana* thylakoid membranes certain spots were suspected to be phosphorylation products by their pI value. Enrichment of phosphopeptides by immobilised metal affinity chromatography and examination of such spots by ESI-MS and MALDI-TOF resulted in identification of phosphorylation sites in a variety of PS II supercomplex proteins. (Vener et al., 2001)

Materials and Methods

1.) Chemical reagents, antibodies, consumables and hardware

Unless otherwise stated chemicals, antibodies, consumables and hardware were purchased from Amersham Biosciences (Freiburg), Biomol (Hamburg), Bio Rad (München), Carl Roth (Karlsruhe), Eppendorf (Hamburg), Fluka (Taufkirchen), Merck (Darmstadt), Millipore (Eschborn), PALL (Dreieich), Quiagen (Hilden) and Sigma Aldrich Chemie (Taufkirchen).

Antibody P25K was a generous donation from A. Boschetti, Bern (Switzerland).
Antibodies anti-18.1, anti-17.2, anti-PsaD and anti-PsaF were kind donations from J.D. Rochaix, Geneve (Switzerland).
Antibody anti-D1 was a gift from U. Johanningmaier, Halle.

2.) Cultivation of *Chlamydomonas reinhardtii*

2.1.) Cultivation in liquid medium

Different strains of the green alga *Chlamydomonas reinhardtii* were cultivated on 1,5 % TAP or HSM Agar plates. For preparation and fractionation of thylakoids cells were grown in liquid TAP

medium (see table 1) under shaking (100 rpm) at 25°C and 80 µE up to a cell density of about 2 x 10^6 cells/ml.

The growth media had the following compositions:

	TAP	**HSM**
H_2O	972 ml	967.5 ml
Tris	2,42 g	-
[1]Trace	1 ml	1 ml
[2]1 M phosphate-buffer for TAP, pH 7	1 ml	-
[3]2 x Phosphate-buffer for HSM	-	6.5 ml
[4]4 x Beijerinck salts	25 ml	25 ml
Acetic acid	about 1 ml (pH 7)	-

Table 1: Composition of growth media

Composition of stock solutions:

Trace elements:

[1]Trace	
BO_3H_3	11.4 g
$ZnSO_4 \cdot 7H_2O$	22.0 g
$MnCl_2 \cdot 4H_2O$	5.06 g
$FeSO_4 \cdot 7H_2O$	4.99 g
$CoCl_2 \cdot 6H_2O$	1.61 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$Mo_7O_{24}(NH_4)_6 \cdot 4H_2O$	1.1 g

Table 2: Composition of trace elements solution

The above components were dissolved in the order indicated and heated to 100°C. 50 g EDTA were dissolved in 250 ml H_2O_{dd}, added to the solution and heated to 100°C. After cooling to 80 - 90°C the pH was adjusted to 6.5 - 6.8 (KOH). The solution was filled up to 1 l and left to stand at room temperature in a 2 l Erlenmayer flask loosely stoppered with cotton until a rust-coloured precipitate formed. Then the solution was filtered several times through three layers of filter paper under suction. (Harris, 1989)

[2,3]Phosphate-buffers for TAP and HSM media (1 l):

	[2]Phosphate-buffer for TAP	**[3]2 x Phosphate-buffer for HSM**
K_2HPO_4	108 g	28.8 g
KH_2PO_4	56 g	14.4 g

Table 3: Composition of Phosphate-buffers

[4]4 x Beijerincks salts (1l):

[4]4 x Beijerincks salts	
NH_4Cl	16 g
$CaCl_2$	2 g
$MgSO_4$	4 g

Table 4: Composition of [4]4 x Beijerincks salts

2.2.) Cultivation on Agar plates

Cultures of *Chlamydomonas reinhardtii* were streaked on 1.5% agar plates and cultured at a constant light intensity of 80 µE at 25°C. The cultures were transferred to fresh plates every three to four weeks.

For the preparation of 1.5% agar-plates 15 g agar agar were added to 1 l HSM or TAP medium (Table 1). The suspension was autoclaved and after slight cooling was poured into plastic petri dishes. For agar plates containing zeocin 25 mg of zeocin were added per liter.

3.) Determination of cell density

Cell density was defined microscopically by counting in a Neubauerchamber.

4.) Preparation of thylakoids

Composition of buffers for thylakoid preparation:

	H1	H2	H3	H4	H5	H6
Sucrose	0.3 M	0.3 M	1.8 M	1.3 M	0.5 M	-
HEPES-NaOH, pH 7.5	25 mM	5 mM	5 mM	5 mM	5 mM	5 mM
$MgCl_2$	5 mM	-	-	-	-	-
EDTA, pH 8	-	10 mM	10 mM	10 mM	10 mM	10 mM

Table 5: Composition of thylakoid preparation buffers

Thylakoids were prepared as described previously (Chua and Bennoun, 1975). All preparation steps and centrifugations were performed on ice or 4°C.

For preparation of thylakoids 1 - 2 l of liquid cell cultures of *Chlamydomonas reinhardtii* at a cell density of about 2 x 10^6 cells/ml were used. The cells were sedimented in a GS3 Rotor in a Sorvall RC-5B centrifuge, using two to four 500 ml centrifuge bottles, for 5 min. at 5000 rpm. To get rid of leftovers of growth media pellets were washed by resupension in 50 to 100 ml H1 buffer and recentrifugation under the same conditions. After this the cells were resupended in 50 - 100 ml H1 buffer and broken up in the nebuliser at 10 - 15 ψ for cellwall-deficient strains and 80 ψ for other strains. The suspension of broken cell material was then centrifuged in the SS34 rotor at 5000 rpm for 10 min.. The supernatant containing primarily cytosolic components was discarded and the pellet was resuspended in 30 ml H2 and was then centrifuged in the SS34 rotor at 20000 rpm for 10 min.. The supernatant was discarded and the pellet was resuspended in 12 ml H3.

For the preparation of a density gradient the suspension of membranes and organell-components was placed into plastic inserts for SW28 centrifuge tubes and overlayed with 12 ml of H4 and H5 each. Ultracentrifugation of the density gradient was performed in SW28 tubes in a Beckman ultracentrifuge at 24000 rpm for 45 min.. After ultracentrifugation green bands accumulated between the density layers, that contained the thylakoids. The bands were carefully removed with a pasteur pipette and diluted in a fivefold volume of H6 buffer to reduce the sucrose concentration. The thus pelletable thylakoids were then centrifuged in a SS34 rotor at 20000 rpm for 30 min.. The supernatant was decanted and the thylakoids were carefully resuspended in a small volume of H6 buffer.

5.) Fractionation of thylakoids:

The following solutions were used for the fractionation of thylakoids:

	gradient solution 1	gradient solution 2
Sucrose	1 M	0.4 M
HEPES/KOH (pH 7.5)	20 mM	20 mM
β-dodecylmaltoside	0.03 %	0.03 %

Table 6: Composition of gradient solutions for fractionation of thylakoids

For the preparation of the gradient 2 ml of 2 M sucrose were pipetted into a SW40 insert tube for stabilisation of the gradient and carefully overlayed with gradient solution 1 and 2. This step gradient was frozen at -70°C and thawed at 5°C which resulted in the formation of a continuous gradient of 0.4 - 1 M sucrose.

Thylakoid membranes were solubilised in aliquots containing 0.8 mg/ml chlorophyll by incubation on ice with β-dodecylmaltoside at a final concentration of 0.9%. After this the samples were centrifuged in a SS34 rotor at 15000 rpm and 4°C for 10 min.. The supernatant containing the solubilised proteins was layered on top of the continuous sucrose gradient from 0.4 - 1 M sucrose. The gradient was then centrifuged in a SW40 rotor at 35000 rpm and 4°C overnight.

To isolate PSI, specific bands known to be specifically enriched in PSI were recovered from the gradient, or the solubilised thylakoids were fractionated over the whole gradient. Therefore the solubilised thylakoids were removed with a peristaltic-pump through a cannula that was drilled through the insert tube just above the 2 M sucrose cushion and collected in fractions of about 1 ml.

Relevant fractions enriched in PSI were identified by immunodetection with specific antibodies for PsaD and Lhca3 after separation by onedimensional gelelectrophoresis and Western Blot.

6.) Determination of protein concentration

For determination of protein concentration the Bicinchoninic Acid (BCA) method was applied according to the manufacturer´s instructions (Sigma Aldrich Chemie GmbH, Steinheim, Germany). First a set of samples for calibration was prepared. Therefore two samples containing 0 (zero samples), 5, 10 and 15 µl BSA-solution respectively, were filled up with H_2O to 40 µl. 960 µl of the reagent were added, consisting of BCA and copper(II)sulfate in a ratio of 50:1. Depending on the protein content estimated by eye, 1 - 10 µl of the sample were treated in the same way. The probes were well mixed and incubated for 30 min. at 55°C. Measurement of extinction was done at 562 nm with a spectralphotometer. The zero line and all the other values were measured against a zero sample. For determination of protein content the extinction values per µl BSA were calculated from the calibration samples. The average value was used to calculate protein concentrations from the samples containing the unknown probes. (Smith et al., 1985)

7.) Pigment analysis

7.1.) Spectrophotometric determination of chlorophyll concentration

For determination of chlorophyll concentration the method described by Porra was used (Porra et al., 1989). Therefore 10 µl of sample were extracted in 990 µl acetone (80%) by vigorous shaking. Then the samples were centrifuged for 4 min. at room temperature to sediment proteins. Measurement of extinction was performed at wavelengths of 646.6, 663.6 und 750 nm, the zero line and all the other values were determined against acetone (80%). To calculate the chlorophyll concentration for every sample the individual extinction value at 750 nm was subtracted from $E_{663.6}$ and $E_{646.6}$ and the corrected values were used in the following formula for determination of Chl a, Chl b and total chlorophyll:

$$\textbf{[Chl a]} = (0.01225 * E_{663.6} - 0.00255 * E_{646.6}) * \text{dilution factor} * \text{mg/ml}$$

$$\textbf{[Chl b]} = (0.02031 * E_{646.6} - 0.00491 * E_{663.6}) * \text{dilution factor} * \text{mg/ml}$$

$$\textbf{[chl]} = (\textbf{[Chl a]} + \textbf{[Chl b]}) * \text{mg/ml}$$

7.2.) Pigment analysis by high performance liquid chromatography

Samples containing approximately 1 µg chl were mixed with 33 µl of 2-butanol. After phase partitioning 16 µl of NaCl were added under vigorous shaking to intensify polarisation. After centrifugation for 2 min. in a table top centrifuge 17 µl of the upper phase containing the pigments were mixed with 34 µl of acetone (80%) and centrifuged for 1 min.. 20 µl of the pigment solution

were applied to a Chromolith Speed-Rod column (RP 18E, 50 mm x 4.6 mm, Merck, Darmstadt) equilibrated with acetone (80%). The diluted (70%) and the 100% acetone phase were delivered by a PU-1580 pump and mixed with an LG-1580-04 gradient mixer (Jasco, Groß-Umstadt) according to the following program: 70 to 100% acetone during the first 3 min., 100% acetone from 3 to 4 min., 100 to 70% acetone from 4 to 6 min.. The column eluent was monitored with a diode array detector MD-1515 at 440 nm (Jasco, Groß-Umstadt). Peak areas could be calculated into terms of concentration by the Borwin PDA software (Jasco, Groß-Umstadt) using calculation factors obtained by calibration with pigment solutions of known concentration.

8.) *In vitro* phosphorylation

8.1.) *In vitro* phosphorylation with cold ATP

For *in vitro* phosphorylation with cold ATP the following phosphorylation buffer (per 100 ml) was used:

cold phosphorylation buffer	
HEPES-NaOH	1.19 g (50 mM)
sorbitol	1.82 g (100 mM)
$MgCl_2$	0.10 g (5 mM)
NaCl	0.03 g (5 mM)
NaF	10 mM

Table 7: Composition of phosphorylation buffer for *in-vitro*-phosphorylation with cold ATP

For *in vitro* phosphorylation the conditions for state transitions were simulated, that activate a thylakoid-localised LHC kinase. Phosphorylation buffer was added to the thylakoid membranes to adjust to a chlorophyll concentration of 0.1 mg chl / ml and the sample was incubated on ice for 30 min. in the dark. After addition of 500 nmol ATP phosphorylation was induced by incubation at 25°C and a light intensity of 150 µE (Fleischmann et al., 1999).

8.2.) *In vitro* Phosphorylation with γ-ATP^{32}

For *in vitro* phosphorylation of thylakoids with γ-ATP^{32} the following phosphorylation buffer was used:

hot phosphorylation buffer	
Sucrose	100 mM
HEPES-NaOH	50 mM
$MgCl_2$	10 mM
ATP	0.2 mM
NaF	10 mM

Table 8: Composition of phosphorylation buffer for *in vitro* phosphorylation with γ-ATP^{32}

Cells were harvested after reaching the log-phase and put in the dark to induce state 1. Thylakoids were isolated in the dark to prevent activation of the LHC kinase and diluted to a chlorophyll concentration of 0.2 mg chl / ml. In the dark 20 µl of an ATP-mixture consisting of 10 µl 20 mM ATP and 10 µl γ-ATP^{32} (20 µ Ci) were added to the diluted thylakoids. After application of a light intensity of 100 µE the phosphorylation reaction was started. Samples were taken after ten and

twenty minutes. Therefore an aliquot containing 20 µg chlorophyll was centrifuged for two min.. The supernatant was discarded and the pellet was resuspended in 100 µl phosphorylation buffer.

9.) 2-dimensional gelelectrophoresis

9.1.) Protein precipitation (chloroform / methanol)

All steps of procedure for protein precipitation and rehydration were performed at room temperature in 1.5 ml-tubes. Centrifugation steps were performed in a benchtop centrifuge. For protein precipitation samples containing 200 µg protein were used.

The samples were filled up with water to 100 µl. To remove non-protein membrane components the sample was extracted with 400 µl methanol under vigorous shaking and centrifuged for 10 s. The pellet was discarded after the supernatant had been carefully transferred to a new tube. After addition of 100 µl of watersaturated chloroform the sample was again shaken and centrifuged for 10 s. Shaking with 200 µl H_2O caused a white, cloudy fallout. To separate phases the sample was centrifuged for 10 s. The protein phase thus became clearly visible in the boundary layer between the organic and the aqueous phase. The upper phase was carefully removed and discarded. To purify the protein the lower chloroform phase containing the protein was shaken with 300 µl of methanol and then the protein was sedimented by centrifugation for 2 min.. The supernatant was carefully removed and discarded. This purification step was repeated two times with one ml 95% methanol. The pellet was Speed-Vac dried for about 15 min.. (Wessel and Flugge, 1984)

9.2.) Rehydration + the first dimension (isoelectric focussing)

For rehydration of the dried thylakoid proteins the following buffer was used:

rehydration buffer		
urea	8 M	1.52 g
thiourea	2 M	0.48 g
DTE	30 mM	14.5 mg
CHAPS	4 %	0.13 g
Tris-base	20 mM	126 µl (0.5 M)
bromophenolblue	0.5%	15.8 µl
H_2O		1.26 ml

Table 9: Composition of rehydration buffer

For solubilisation the precipitate was taken up in 380 µl of rehydratisation buffer. After addition of 1.9 µl IPG-buffer und 1.9 µl β-dodecylmaltoside (10%(w/v)) the samples were shaken at room temperature for 1 h. Insoluble particles were sedimented by centrifugation. 350 µl of the supernatant were carefully transfered to another tube and 1.75 µl of IPG buffer were added. After this the samples were pipetted into the groove of a sample tray. The IPG strip (pH 3 – 10) was overlayed upside down and covered with paraffin. For rehydratisation the samples were incubated for 12 h at 20°C. Then isoelectric focussing was performed at 15°C. According to the following program the voltage was raised over the next 10 to 12 hours to 8000 Volt in a stepwise process (step and hold), applying a constant current of 50 µA to each sample:

	voltage (V)	time (h)
step 1	300	0.25
step 2	500	0.5
step 3	1000	0.5
step 4	1500	1
step 5	3000	1
step 6	8000	8

Table 10: Step´n´hold-program for IEF

IEF was terminated after 60000 Vh.

9.3.) Equilibration for second dimension

For equilibration of IPG strips the following buffers were used:

	equilibration buffer 1		equilibration buffer 2	
Tris-HCl, pH 6.8	50 mM	10 ml (0.5 M)	50 mM	10 ml (0.5 M)
urea	6 M	36 g	6 M	36 g
glycerine (87%)	30 %	30 ml	30 %	30 ml
SDS	2 %	2 g	2 %	2 g
DTE	2 %	2 g	-	-
iodineacetamide	-	-	2.5 %	2.5 g
bromophenolblue	-	-	0.5 %	0.5 ml
H_2O_{dd}		ad 100 ml		ad 100 ml

Table 11: Composition of equilibration buffers

To make the focussed proteins separatable by SDS-PAGE by denaturing and reducing chemicals, IPG-strips were treated subsequently with the two equilibration buffers: First the strips were incubated in equilibration buffer 1 for 12 min., then in equilibration buffer 2 for 5 min.. Between the two incubations the strips were washed with H_2O_{dd}. The equilibrated strips were placed on top of the SDS-gels and fixed with 0.5% agarose.

9.4.) The second dimension (SDS-PAGE)

For SDS-PAGE 1 mm thick gels (20 x 20 cm) containing 13% acrylamide were cast. Glassplates were thoroughly cleaned with ethanol and H_2O_{dd} and assembled with the BioRad system. The casting solution was prepared according to the following recipe:

Casting solution for one acrylamide gel (13%):

Tris-HCl, 1.5 M, pH 8.8	9.25 ml
acrylamide/PDA, 30% / 0.8%	16 ml
H_2O_{dd}	11.54 ml
sodium-thiosulfate (5%)	181 µl
TEMED	14 µl
APS(10%)	140 µl

Table 12: Composition of acrylamide gels

The polymerisation reaction was started with the addition of TEMED and APS and the solution was pipetted between the glass plates without any delay. The solution was overlayed with isopropanol and left to polymerise for about one hour. After this the isopropanol layer was cast away and the gel was washed with H_2O_{dd}.

For gelelectrophoresis the following **running buffer** (1l, 10 x) was used for the upper and the lower reservoir:

running buffer	
SDS	10 g
Tris-Base	30 g
glycine	144 g

Table 13: Composition of running buffer for SDS-PAGE

10.) 1 – dimensional gel electrophoresis

10.1.) SDS – PAGE (Laemmli)

For onedimensional gelelectrophoresis (Laemmli) stacking gels and separation gels with the following compositions were used

	separation gel	**stacking gel**
acrylamide (30%, 0.8% PDA)	13.0 ml	1.5 ml
lower Tris (1.5 M, pH 8.8)	7.6 ml	-
upper Tris (0.5 M, pH 6.8)	-	2.26 ml
H_2O	7.9 ml	5 ml
EDTA (0.5 M, pH 8)	121 µl	36 µl
sucrose (2 M)	1.28 ml	-
TEMED	7.8 µl	9 µl
APS (50 %)	75.5 µl	18 µl

Table 14: Compositions of separation and stacking gels for SDS-PAGE (Laemmli)

The loading buffer was of the following composition:

loading buffer	
glycerine (87%)	5.74 ml
β-mercaptoethanol	2.5 µl
Tris (0.5 M, pH 6.8)	6.25 ml
DTT	1.54 g
bromophenolblue (0.5 %)	1 ml
SDS	3.1 %

Table 15: Composition of loading buffer for onedimensional SDS-PAGE (Laemmli, Schaegger)

The samples were mixed with loading buffer 1:1 (but at least 15 µl of loading buffer were used) and incubated for 15 min. at 60°C.

The same running buffer and electrophoresis conditions were applied as described for 2-DE (9.4.).

10.2.) SDS-PAGE (Schaegger)

For 1-dimensional gelelectrophoresis (Schaegger) separation-, stacking and spacergels were cast with the following compositions:

	separation gel	spacer gel	stacking gel
acrylamide (30%, 0.8% PDA)	20.8 ml	4.88 ml	0.8 ml
*gelbuffer**	13.0 ml	5.0 ml	1.55 ml
glycerine	5.98 g		
H_2O	ad 39 ml	ad 15 ml	ad 6.25 ml
TEMED	13 µl	5 µl	3 µl
APS (50 %)	50 µl	25 µl	15 µl

gel buffer: 3 M Tris, **pH 8.45, 0.3 %(w/v) SDS, for 1 l: 363.3 g Tris-Base, 3.0 g SDS*

Table 16: Compositions of separation, spacer and stacking gel for SDS-PAGE (Schaegger)

For electrophoresis the following electrode buffers were used:

	anode buffer		kathode buffer	
Tris	0.2 M	24.22 g	0.1 M	12.11 g
tricine	-	-	0.1 M	17.92 g
SDS	-	-	0.1 % (w/v)	1.0 g
HCl	to pH 8.9		-	-

Table 17: Composition of electrode buffers for SDS-PAGE (Schaegger)

Loading buffer was used as described for SDS-PAGE (Laemmli)(see II14.1.), the running conditions were set as described for 2-DE (see II7.4.).

10.3.) Deriphat-PAGE

To separate thylakoid proteins in their native state the Deriphat-system according to (Peter et al., 1991) was used.

The acrylamide-gel was made of the following constituents:

Gel for Deriphat-PAGE	
H_2O	17.55 ml
gelbuffer	2.5 ml
acrylamide-solution[*]	5.85 ml
APS (10%)	0.4 ml
TEMED	20 µl

[*] *acrylamide-solution: 33.5% acrylamide, 0.3% N,N´ - methylenbisacrylamide*

Table 18: Composition of acrylamide-gel for Deriphat-PAGE

An aliquot of thylakoid proteins containing 20 µg chlorophyll was solubilised with β – dodecylmaltoside in a ratio β-DM : chl = 10 : 1 for 1 min. on ice and then centrifuged for 1 min.. The supernatant was loaded onto the gel. Electrophoresis was performed with a constant voltage of 100 Volt for about two hours.

The following reservoir and gel buffers were used:

	reservoir buffer (10 x)	gel buffer (10 x)
Tris-base	15 g/l (124 mM)	15 g/l (124 mM)
glycine	72 g/l (960 mM)	36 g/l (480 mM)

Table 19: Composition of reservoir and gel buffer for Deriphat-PAGE

11.) Western blot analysis

11.1.) Western protein transfer

Protein transfer was performed either by semi-dry-blotting or wet-blotting.

For protein transfer using the semi-dry-blot-apparatus 1 x running buffer containing 20 % methanol was used.

For wet-blotting the following blotting buffer was used:

wet-blotting buffer	
CAPS	20 mM
Methanol	20 %
NaOH	to pH 11

Table 20: Composition of wet-blotting buffer

For both protein transfer procedures the gel was placed between 4 layers of filter paper dipped in blotting buffer. A nitrocellulose or a PVDF membrane was placed directly on the gel on its anode side. The separated proteins were transferred at a constant amperage between 80 - 400 mA for 12 to 2.5 hours.

11.2.) Ponceau Rouge staining

After protein transfer the membranes were stained with Ponceau Rouge to test for the efficiency of the blotting procedure and to fix the protein bands. Therefore the membranes were panned in Ponceau Rouge for 1 min.. After removal of the solution with H_2O_{dd} the red stained protein bands were visible.

Composition of Ponceau Rouge solution:

Ponceau Rouge	
Ponceau Rouge	0.5 g
Acetic acid	1 ml
H_2O_{dest}	ad 100 ml

Table 21: Composition of Ponceau Rouge solution

11.3.) Immunodetection

11.3.1.) Incubation of blots with antibodies

For incubation of the blots with antibodies the following buffer was used:

PBS-buffer(1 l, 10 x)	
NaCl	80.1 g
KH_2PO_4	2 g
Na_2HPO_4	11.2 g
KCl	2 g

Table 22: Composition of PBS-buffer

To saturate the blotting membrane and to prevent unspecific binding of antibodies the membrane was incubated for 30 - 60 min. in PBS buffer containing 2% low fat milk powder (Milbona, UK). The incubation with primary antibodies (in PBS-milk-powder-buffer 1:1000) was done for at least one hour. After this the blot was incubated again in PBS for 2 x 10 min. to remove unspecifically bound antibodies. Then the secondary antibody anti-IgG(rabbit)-peroxidase was applied in PBS-milk-powder-buffer (1:10000) for about one hour. After this unspecifically bound antibodies were again washed off by incubation for 2 x 10 min. in PBS-milk-powder-buffer.

11.3.2.) Detection of bound antibodies by chemiluminescence

The following solutions were used per blot:

solution A:

13.3 µl p-coumaric acid (90 mM, 14 mg/ml in DMSO) in 3 ml Tris (100 mM, pH 8.5) + 1.66 µl H_2O_2

solution B:

30 µl 3-aminophthalhydrazine (luminol, 250 mM, 44 mg/ml in DMSO) in 3 ml Tris

The blot was incubated in solutions A and B for one minute at room temperature. Then the blot was dried two times with paper towels and exposed to an X-ray-film (Amersham Biosciences, Little Chalfont, UK). The film was processed in the developer machine (AGFA, Suhl, Germany).

12.) In gel protein staining

12.1.) Silver staining

The following solutions were used for silver staining of one gel (in 250 ml H_2O_{dd}):

fixing solution (10% acetic acid, 40 % ethanol), **sensitiser** (75 ml ethanol, 0.5 g sodium thiosulfate, 17 g sodium acetate), **silver solution** (0.625 g silver nitrate), **developer** (6.25 g sodium carbonate, 100 µl formaldehyde), **stop solution** (3.65 g titriplex III)

The gel was incubated for 30 min. each in fixing solution and sensitiser. Then the Gel was washed for 3 x 5 min. in H_2O_{dd} and stained for 20 min. in silver solution. Unbound silver nitrate was removed by incubation in H_2O_{dd} for 2 x 1 min.. The bound silverions became visible by incubation with developer after about 4 min., with formaldehyde added to the developer solution shortly before incubation. Developing was terminated by incubation with stop solution.

12.2.) Coomassie Brilliant Blue staining

For Coomassie staining staining and destaining solutions with the following compositions were used (per l):

	staining solution	destaining solution
ethanol	400 ml	300 ml
acetic acid	100 ml	100 ml
H_2O_{dd}	500 ml	600 ml
Coomassie Sigma Brilliant Blue G	1 g	-

Table 23: Compositions of Coomassie staining and destaining solutions

The gel was incubated in the staining solution for about one hour. Destaining was performed overnight with occasional renewing of the destaining solution.

12.3.) Colloidal Coomassie staining

Colloidal Coomassie staining solution was of the following composition (250 ml):

Colloidal Coomassie	
ammonium sulfate	42.5 g
methanol	85 ml
phosphoric acid (85 %)	9 ml
Coomassie G - 250	0.25 g
H_2O	ad 250 ml

Table 24: Composition of Colloidal Coomassie staining solution

Methanol was filled up with H_2O to a volume of about 200 ml. Ammonium sulfate was dissolved stepwise under heating. Then the phosphoric acid was added and Coomassie was added carefully in a stepwise procedure under heating .

The gel was stained in the solution for one hour, and then destained for 30 min. with methanol (30%).

13.) Proteolytic in-gel-digest

Relevant spots were excised with a sterile scalpel and destained for 8 min. with 300 - 500 µl of a 1:1 mixture of 30 mM kaliumferricyanide and 100 mM sodium thiosulfate. If necessary, destaining was repeated with 100 µl of the same mixture for 1 min.. Then the gel slices were washed four times for 1 min. with 1 ml H_2O_{dest} and shrunk for 20 min. using 60 % acetonitrile in water. The supernatant was discarded and the spots were Speed-Vac-dried for 15 min. and rehydrated for 20 min. with NH_4HCO_3. The procedure of shrinking and rehydrating was repeated two more times. The third rehydration was performed on ice with 0.5 µg trypsin added. After removal of supernatant in gel proteolytic digest was performed for 12 hours at 37°C with 50 µl 50 mM NH_4HCO_3. After 12 hours the supernatant was removed and kept for further analysis and peptides were eluated from the gel pieces for two to three hours using 100 µl 60 % Acetonitril. The extracts were combined and kept at -80°C for further analysis.

14.) Mass spectrometry (LC-ESI)

Analyte sampling, chromatography and production and acquisition of MS and MS-MS data were performed online by using fully automated instrument functions. Solvents were purchased from Merck and were of LC grade.

14.1.) Analyte sampling

The combined extracts from the trypsin digest were lyophylised and resuspended in 40 µl of a 5:90:5 methanol-water-formic acid solution and vortexed for 20 min.. A Famos 48-well plate autosampler (LC-Packings, Amsterdam, The Netherlands) was used to sample the tryptic digests in batch mode with a user-defined program to withdraw 1 µl from each sample.

14.2.) Liquid chromatography

The aqueous phase (A)(0.1% [vol/vol] formic acid in 5:95 acetonitrile-water) and the organic phase (B)(0.1% [vol/vol] formic acid in 80:20 acetonitrile-water) were delivered by an Ultimate (LC-Packings) micropump according to the following program: 5% B in the first 8 min., 5 to 50% B from 8 to 38 min., 50 to 95% B from 38 to 39 min., 95% B from 39 to 49 min., 95 to 5% B from 49 to 50 min., and 5% B from 50 to 75 min.. The peptide mixture was fractioned on an LC-Packings PepMap C_{18} column (75-µm [inner diameter] by 150 mm) with a 3-µm particle size and a 100-Å-pore diameter (NAN-75-15-03-PM). The column eluent was monitored at 214 and 280 nm with a rapid-scanning spectrophotometer equipped with a 3-nl flow cell (LC-Packings UZ-N10 160015).

14.3.) Electrospray ionisation

The interface between the liquid phase and the gas phase was provided by a fused silica needle that had a 75-µm inner diameter, a length of approximately 12 cm, and a 30-µm aperture (FS 360-75-30; New Objective, Woburn, Mass.) and that was mounted on a nano-electrospray ionisation source

(New Objective). Atmospheric pressure electrospray ionisation was accomplished by applying a voltage difference of +1.3 kV across the fused silica needle. The aperture of the fused silica needle was positioned 1 to 2 mm from the opening of the ion transfer capillary and slightly off axis to minimise the entrance of nonvaporised solvent into the LCQ Deca XP ion trap mass spectrometer (Thermofinnigan, San Jose, Calif.). No sheath gas was used.

14.4.) Ion scan

Most mass spectra were acquired by using the repetitive "triple-play" sequence as recommended by the manufacturer, consisting of a full scan event for ions with mass-to-charge ratios of 400 to 1200, a zoom scan event acquired within a mass-to-charge ratio window of 10 units centered around the chosen ion, and an MS-MS scan event. Ions were selected for the zoom scan and for the MS-MS scan automatically by using instrument software in the data-dependent manner, whereby ions of sequentially decreasing abundance were chosen and two scan events were allowed for any given ion in a 3-min time window. For complex samples, the zoom scan event was omitted and replaced with two MS-MS scan events. The tolerance for the selection of the MS-MS precursor ranged from 1.5 to 3.0 *m*/*z* (low to high *m*/*z*).

14.5.) Data acquisition

The measured MS-MS spectra were matched with tryptic peptide amino acid sequences from a small *C. reinhardtii* database consisting primarily of PSI or PSII polypeptide sequences (about 90 entries), from the translated Kazusa EST database (Asamizu et al., 2000; Asamizu et al., 1999), from the EST database of the *Chlamydomonas* genome project (A. Grossman, J. Davies, N. Federspiel, E. Harris, P. Lefebvre, C. Silflow, D. Stern, and R. Surzycki, unpublished data), or from a genomic database (version 1.0 assembly). In addition, the data sets were partially compared with the genomic sequences for the presence of new LHC polypeptides. All databases were in the FASTA format. Cys modification by carbamidomethylation (+57 Da) was taken into account. Raw MS-MS data files that had a minimum total ion current of 10^5 and contained 15 or more fragment ions were selected. Known contaminants were filtered out. Computational analysis was done on a Dell personal computer. The tolerance window set for the grouping of raw MS-MS data files into input files for the Finnigan Sequest/Turbo Sequest software (revision 2.0; ThermoQuest, San Jose, Calif.) was set to 1.4 atomic mass units.

The Sequest algorithm was used to quickly identify and retrieve database sequences having at least one tryptic end, having a theoretical mass within ±1.25 atomic mass units of that measured for the precursor ion, and having a theoretical y- and b-ion fragmentation profile with a high degree of similarity to the experimentally measured MS-MS spectrum.

14.6.) Data evaluation

The similarity between a measured MS-MS spectrum and a theoretical MS-MS spectrum, reported as the cross-correlation factor (X_{corr}), and the difference between the unit-normalised X_{corr} values of the first- and second-ranked sequences (ΔCorr) provided the preliminary criteria for assigning amino acid sequences to experimental MS-MS spectra. Sequences are reported here if the X_{corr} values calculated for the measured and theoretical MS-MS spectra were equal to or above 1.5, 2.25, or 3.5 for singly, doubly, or triply charged precursor ions, respectively, and if the ΔCorr value exceeded 0.1 within the background of the Kazusa EST database (Asamizu et al., 2000; Asamizu et al., 1999) or the genomic database.

14.7.) N termini database

Since the N termini of the mature proteins are not yet clear, a database subset containing sequences of the *lhca* or *lhcb* gene products from *Chlamydomonas* was created. This database contains amino acid sequences where the first N-terminal amino acids of respective gene products were excised in a stepwise manner. This step was repeated 45 times to create different N-terminal sequences. This database, containing different N termini, allowed us to search for N-terminal peptides and also carbamylation.

15.) Mutant screen by random insertional mutagenesis with the *ble*-gene

15.1.) Plasmid amplification

15.1.1.) Preparation of competent cells

The following medium was used for cultivation of *E.coli* (per l):

LB-Medium	
NaCl	10 g
tryptone	10 g
yeast extrat	5 g

Table 25: Composition of LB-medium

For preparation of competent cells a culture of *Escherichia coli* grown in LB-Medium was harvested at an optical density of 0.7. Optical density was measured in a spectralphotometer at 600 nm against LB-medium. The cells were washed twice by centrifugation in a GS3 rotor at 5000 rpm for 10 min. and resuspension in 500 ml H_2O_{dd}. In the second run the cells were resuspended in 250 ml H_2O_{dd}. After another centrifugation using the same parameters the cells were resuspended in 10 ml 10 % glycerole (in H_2O_{dd}) and then centrifuged in a SS34 rotor at 5000 rpm for 10 min.. Then the pellet was resuspended in 2 ml 10 % glycerol (in H_2O_{dd}).

15.1.2.) Transformation of *E . coli* by electroporation

The following medium was used:

SOC	
Bacto tryptone	2%
Bacto yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
MgCl	10 mM
$MgSO_4$	10 mM
glucose	20 mM

Table 26: Composition of growth medium for *E. coli*

1 µl of plasmid psp115 was added to 40 µl of cells and elctrotransformed at 2500 V. The cells were resuspended in 800 µl SOC (sterile) and after incubation at 37°C for 30 min. 50 µl of cells were streaked on an LB Amp plate. The next day a colony was picked and grown in 30 ml of LB medium containing 0.01% Ampicillin.

15.1.3.) Plasmid isolation

Plasmid isolation was performed with the HiSpeed Plasmid Midi Kit (Quiagen). The following buffers were used (per l):

	P1	P2	P3	QBT	QC	QF
Tris-base	6.06 g					6.06 g
EDTA	3.72 g					
RNase	100 mg					
HCl	ad pH 8.0					ad pH 8.5
NaOH		8.0 g		ad pH 7.0	ad pH 7.0	
SDS (20%)		50 ml				
potassium acetate			294.5 g			
glacial acetic acid			ad pH 5.5			
NaCl				43.83 g	58.44 g	73.05 g
MOPS				10.46 g	10.46 g	
isopropanol				150 ml	150 ml	150 ml
Triton X-100 (10%)				15 ml		

Table 27: Composition of plasmid isolation buffers

30 ml of transformed *E.coli* cells were centrifuged in a SS34 rotor for 5 min. at 6000 rpm. After resuspension of the pellet in 4 ml P1 and addition of 4 ml P2 the suspension was incubated at room temperature for 5 min.. After addition of 4 ml P3 the resuspended cells were incubated on ice for 15 min. with shaking from time to time. After centrifugation in the SS34 rotor at 11000 rpm for 30 min. the cell suspension was filtered and applied to a column equilibrated with 5 ml QBT. The column was washed two times with 10 ml QC. DNA was eluated with 5 ml QF and collected in a 15 ml Corex tube. After addition of 3.5 ml isopropanol the solution was mixed well and centrifuged in a SS34 rotor at 12000 rpm for 30 min.. The supernatant was discarded and the pellet was dissolved in 2 ml 70 % ethanol. After centrifugation in the SS34 rotor at 12000 rpm for 15 min. the supernatant was discarded and the pellet was resuspended in 80 µl H_2O. The absorption of a 1:100 - dilution of a DNA-sample was measured at 260 and 280 nm against H_2O. To determine the DNA-concentration the following formula was used:

$$A_{260} / A_{280} * 50 * 100 = [DNA]$$

15.2.) Nuclear transformation of *Chlamydomonas reinhardtii* with the *ble*-gene using the glass-beads method

For the generation of high-light-resistent LHC-assembly-mutants 300 ml of cell culture of strain wildtype CW15 grown in TAP medium were centrifuged in the log phase. Cells were resuspended in 3 ml TAP medium and thoroughly shaken in a test tube containing sterile glass pearls and 1 µg of plasmid psp115 (Stevens et al., 1996) for 15 sec.. (Kindle, 1990) The transformed cells were streaked on HSM or TAP agar plates containing 25 mg/l zeocin®. The plates were cultured at 25°C and a light intensity of 300 µE .

16.) Preparation of plastocyanin

Composition of resuspension and elution buffer:

	resuspension buffer	elution buffer
Tricin / KOH pH 7.6	0.02 M	0.02 M
KCl	0.02 M	0.6 M

Table 28: Composition of buffers used in preparation of plastocyanin

Plastocyanin was isolated from a 5-l-culture of *Chlamydomonas reinhardtii* grown in TAP medium to a density of 2 x 10^6 cells/ml. Cells were sedimented in a GSA rotor at 5000 rpm for 5 min. and resuspended in 100 ml isolation buffer. Cells were frozen at -20°C, thawed and then frozen again at

-20°C with liquid nitrogen. After thawing the broken cell material was sedimented in the SS34 rotor at 20000 rpm for 20 min..

The supernatant was applied to a column containing Diethylaminoethyl(DEAE)-cellulose for anion exchange chromatography at a flow rate of 1 ml/min.. Elution was performed with a 1:1 gradient of resuspension and isolation buffer at 1 ml/min.. Fractions of 2 ml were collected and tested for plastocyanin by addition of 3 µl 0.5 mM potassium hexacyanoferrate III. After addition of potassium hexacyanoferrate III peaks at 597 nm in the photospectrometer indicate plastocyanin in its oxidised form. Fractions containing plastocyanin were combined and applied to a Sepharose column using the same buffers and flow rates as for anion exchange chromatography with DEAE-cellulose. Fractions that turned blue after addition of 3 µl potassium hexacyanoferrate III were concentrated to 0.33 mM with centricon tubes.

17.) Oxygen uptake assay

Composition of reaction buffer:

reaction buffer	
Tricin	160 mM
KCl	160 mM
$MgCl_2$	24 mM

Table 29: Composition of reaction buffer for oxygen uptake assay

Composition of assay mixture (1 ml):

oxygen uptake assay	
NaN_3 (20 mM)	10 µl
NH_4Cl (200 mM)	10 µl
β-dodecylmaltoside (10%)	5 µl
Na-ascorbate (500 mM)	10 µl
DCPIP (10mM)	10 µl
methyl viologen (5mM)	10 µl
DCMU (6mM)	5 µl
reaction buffer	125 µl
thylakoids	≡ 10 µg Chl
plastocyanin (0.33 mM)	3 µl
H_2O_{dest}	**ad 1 ml**

Table 30: Composition of assay mixture for oxygen uptake assay

Gross oxygen uptake was measured at 20°C and atmospheric pressure in the liquid phase with a Clark type polarographic oxygen sensor electrode using the oxy-lab® System (Hansatech, King´s Lynn, UK). The assay mixture was prepared by filling the cuvette with H_2O and then stock solutions, thylakoids and reaction buffer as listed in the table above from top to bottom. Under constant stirring of the assay mixture light intensities of 10, 20, 40, 80, 160, 320, 640 and 1000 µE were applied with the LED light source LH11 MK2 (Hansatech, King´s Lynn, UK) at a wavelength of 660 nm. For each light intensity oxygen uptake was measured for 90 sec..

The thylakoids employed in the oxygen uptake assay had the following chlorophyll and protein contents (µg/µl)(see M&M, 7.1):

	wildtype	S2	cbs3
chl	1.75	1.0	1.6
protein	10.0	8.4	13.3
protein : chl ratio	5.7	8.4	8.3

Table 31: Chl and protein content of thylakoids employed in oxygen uptake assay.

18.) Measurement of 77 K fluorescence

From thylakoid membranes resuspended in 20 mM HEPES pH 7.5 and 60% glycerol to a density equivalent to 5 µg/ml chlorophyll low-temperature (77 K) fluorescence emission spectra were recorded using a Fluoromax-2 spectrofluorometer (Instruments S.A. Inc., Munich, Germany).

Results

1.) 2-DE separation of LHCPs

1.1.) Reproducible high-resolution 2-DE analysis of thylakoid membrane proteins

High-resolution separation of thylakoid membrane proteins is a crucial step towards identifying proteins of the light harvesting complexes of *Chlamydomonas reinhardtii.* For resolution of membrane proteins on 2-DE solubilisation and fractionation of the sample appear to be very critical (Molloy et al., 2000). Chloroform/methanol precipitation to separate thylakoid proteins from pigments and lipids (Wessel and Flugge, 1984) proved a very efficient way to recover polypeptide constituents from thylakoids isolated by density ultracentrifugation (Chua and Bennoun, 1975). Of the protein applied to precipitation only 4 % could be determined in the chloroform/methanol supernatant by the bicinchoninic acid method (Smith et al., 1985), whereas no protein could be found in the water/methanol fraction, indicating that the precipitation procedure does not lead to a significant loss of proteins. Also thiourea could be confirmed to be a crucial ingredient to the solubilisation buffer, as described before (Pasquali et al., 1997). Resolution and reproducibility could also significantly be improved by addition of the nonionic detergent β-dodecylmaltoside to the solubilisation buffer. Finally the solubilisation method including the zwitterionic detergent CHAPS (Rabilloud et al., 1997) and thiourea resulted in reproducible high resolution twodimensional protein maps of *C. reinhardtii* thylakoids and PS I preparations (Fig. 4, Fig. 5;

(Hippler et al., 2001). Averaging 15 silverstained gels, about 80 % of the 350 - 400 detected protein spots were fully reproducible.

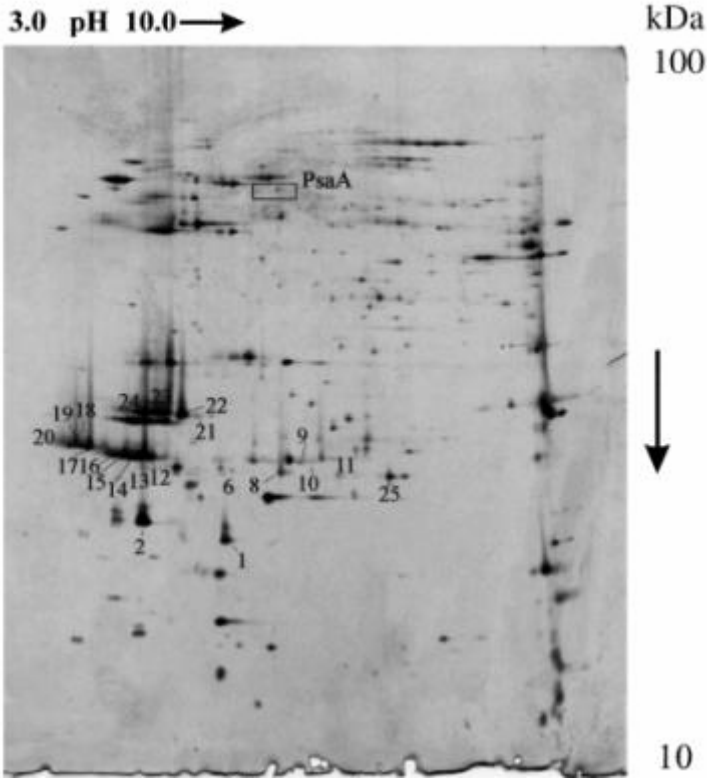


Fig. 4: Hydrophobic proteins such as the LHCPs can be separated by twodimensional gel electrophoresis (2-DE). 2-DE separated wild-type thylakoids (200 µg protein) were analysed by silver staining. The boxed region represents protein spots corresponding to the PsaA polypeptide as revealed from immuno-blot analysis using anti-PsaA antibodies.

1.2.) Identification of LHC proteins by immunoblotting

The high resolution of these protein maps allowed identification of the hydrophobic transmembrane spanning LHC proteins using immunobloting and mass spectrometry. The immunoblot analysis of 2-DE separated wild-type thylakoids and PSI particles using anti-LHCI (anti18.1 (LHCa4); Bassi et al., 1992) and anti-LHCII antibodies (anti-P25K; Michel *et al*., 1983) are shown in Figures 6 and 7, respectively.

1.2.1.) Identification of LHCI proteins by immunoblotting

For further characterisation of LHCI proteins, enriched PSI particles were separated by 2-DE and analysed by silver staining and immunoblotting (Fig. 5). The immunoblots were probed with LHCI

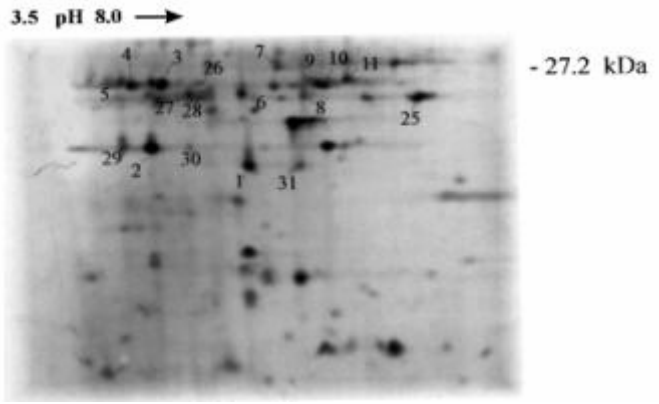


Fig. 5: Separation of PSI particles by 2-DE. 2-DE separated PSI particles (100 µg protein) were analysed by silver staining.

antibodies anti-18.1, anti-17.2, anti-15.1 and anti-14.1. Antibodies anti-15.1 and anti-14.1 were raised against synthetic polypeptides resembling the first 16 N-terminal amino acids of mature p14.1 (Lhca3) and p15.1 (Lhca5). These peptide sequences are unique to these LHCI proteins so

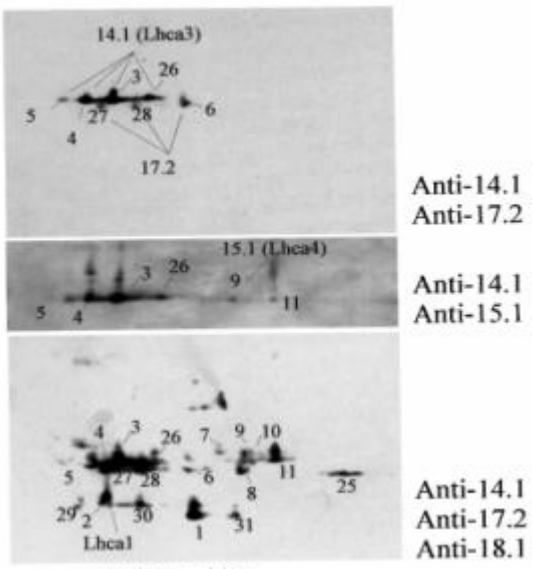


Fig. 6: 2-DE separated PSI particles (100 µg protein) were analysed by immuno-blotting using LHCI specific antibodies (anti-14.1; anti-15.1; anti-17.2 and anti-18.1).

that the antibodies should specifically recognise the corresponding proteins but not other members of the LHC protein family. When the anti-14.1 peptide antibodies are used to probe the Western

blot of 2-DE separated enriched PSI-particles, four spots can be detected (Figure 6, middle panel, spots 3, 4, 5 and 26), that correspond to spots 3, 4, 5 which are also recognised by the anti-18.1 antibodies (Figure 6, lower panel). It is of note that these spots do not differ in size but only in their IEP. On the silverstained 2-DE map of wildtype thylakoids these spots are covered by LHCII proteins (Figure 4) but can be visualised on the silverstained 2-DE map of enriched PSI particles (Figure 5). The second peptide antibody anti-15.1 is less sensitive but recognises two spots on the immunoblot of 2-DE separated PSI particles, which correspond to spots 9 and 11 that are also identified with the anti-18.1 antibodies. Using anti-17.2 antibodies (Bassi et al., 1992), that also react against LHCI proteins, three further spots (6, 27 and 28) are detected on the PSI immunoblot (Figure 6, upper panel). This antibody also strongly cross-reacts with LHCII proteins (Hippler et al., 2001). The fact that no spots corresponding to LHCII proteins are found on the PSI immunoblot indicates that these proteins are absent from the enriched PSI preparation. When this blot is probed with anti-18.1 antibodies, 12 spots, which already had been recognised on the 2-DE immunoblot of thylakoids, are identified. In addition three further minor spots (spots 29, 30 and 31) are detected. The 15 spots are enriched in 2-DE of PSI compared to the 2-DE of thylakoids, which indicates that these proteins co-purify with PSI and suggests that they belong to the antenna that is functionally connected to PSI.

A total of 18 LHCI spots are enriched in 2-DE of PSI compared to the 2-DE of thylakoids, which indicates that these proteins copurify with PSI and suggests that they belong to the antenna that is functionally connected to PSI. The fact that several spots with similar molecular masses but different IPs could be separated, shows that an exact determination of LHCI stoichiometry by onedimensional SDS-PAGE is not possible. To determine the relative stoichiometry of the LHCI subunits, “spot volumes” (using Phoretix 2-D software, Phoretix International, Newcastle-upon-Tyne, UK) of the individual LHCI proteins were evaluated after staining of 2-DE separated PSI

particles with colloidal Coomassie. In the Coomassie stain, LHCI spots, 1, 2, 3, 4, 6, 8, 9, 10 and 25 could be visualised (data not shown). This staining procedure has a higher dynamic range in staining of proteins than silver staining. From this analysis (three independent gels were evaluated) it appears that spot 2, a Lhca1 protein, is the most abundant LHCI subunit. When LHCI spots with the lowest spot volume values (spots 6, 9 and 10), are set to 1, the Lhca1 protein seems to be 50 times more abundant than these low abundant spots. Spots 3 and 4 that correspond to the Lhca3 proteins are each about 30-fold and spots 1, 8 and 25 are about four-fold enriched compared with spots 6, 9 and 10. Analysis of these individual spots by mass spectrometry revealed that nine different lhca gene products could be identified (Stauber et al., 2003). In addition this analysis showed that in each spot one to three distinct *lhca* gene products could be found (Stauber et al., 2003). This clearly documents that relative enrichment of an individual spot can not be correlated to the abundance of one specific *lhc* gene product.

1.2.2.) Identification of LHCII proteins by immunoblotting

Using anti-LHCII antibodies 13 protein spots can be identified in Western blots of 2-DE separated thylakoid membranes (spots 12-24). These spots match with highly abundant protein spots on the silverstained gel (see numbering in Figure 6). The anti-LHCI antibodies (anti-18.1) recognise 12 protein spots on the immunoblot (Figure 7, spots 1-11, 25), which also can be attributed to spots on the silverstained gel. Spots with a higher molecular mass than 27.2 kDa, which are recognised by the anti-18.1 antibodies, correspond most likely to the minor LHCII proteins CP29 and CP26 (see boxed region in Figure 7) since these LHCII proteins have a high homology to p18.1 (Bassi et al., 1992) .

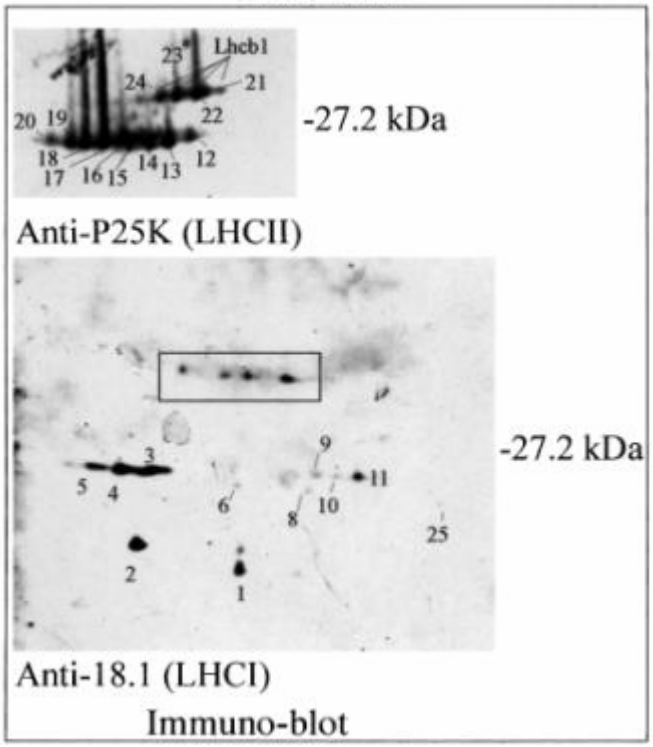


Fig. 7: 2-DE separated wild-type thylakoids (200 µg protein) were analysed by immunoblotting using LHCII (anti-P25K) and LHCI (anti-18.1) -specific antibodies. The boxed region in the lower panel represents protein spots that are recognized by the anti-18.1 antibodies, that correspond most likely to the minor LHCII protein CP29 (Bassi *et al*. 1992).

1.3.) Lhcb protein map

The major spots that were recognised by the anti-LHCII antibody P25K (Fig. 7) were excised from a Coomassie blue stained 2-DE separation of thylakoid membranes (Fig. 8, right panel). Analysis of tryptic digests of the spots 30 and 31, 22 to 24 and 12 to 17 that are diverse in their apparent molecular masses by MS-MS unambiguously identified eight distinct Lhcb proteins (Table 32): Lhcbm1, Lhcbm3, and Lhcbm5 (identified in enriched PSI particles (Stauber et al., 2003)); Lhcbm6, Lhcbm2 or Lhcbm8, and Lhcbm4 or Lhcbm9 (identified from green gels; see below); and CP26 and CP29 (Table 32). Lhcbm1 is LhcII-4, Lhcbm2 is LhcII-3, Lhcbm3 is LhcII-1.3, Lhcbm5 is Lhcb3, Lhcbm6 is Lhcb1 (CabII-1), and Lhcbm8 is CabII-2 (Elrad et al., 2002). For CP26 and CP29, peptides were detected from spots 31 and 30, respectively. Although the Lhcbm proteins are highly similar, peptides could be identified that are redundant but also others that are unique for specific *lhcbm* gene products (Table 32) and thereby distinguish the corresponding protein clearly in the respective protein spot. For spots 22 to 24, the MS-MS data identify specific peptides

corresponding to Lhcbm3. For spots 22 and 23, peptides specific for Lhcbm6 can be identified, whereas for spot 24, peptides specific for Lhcbm4 or Lhcbm6 can be found. The occurrence of the

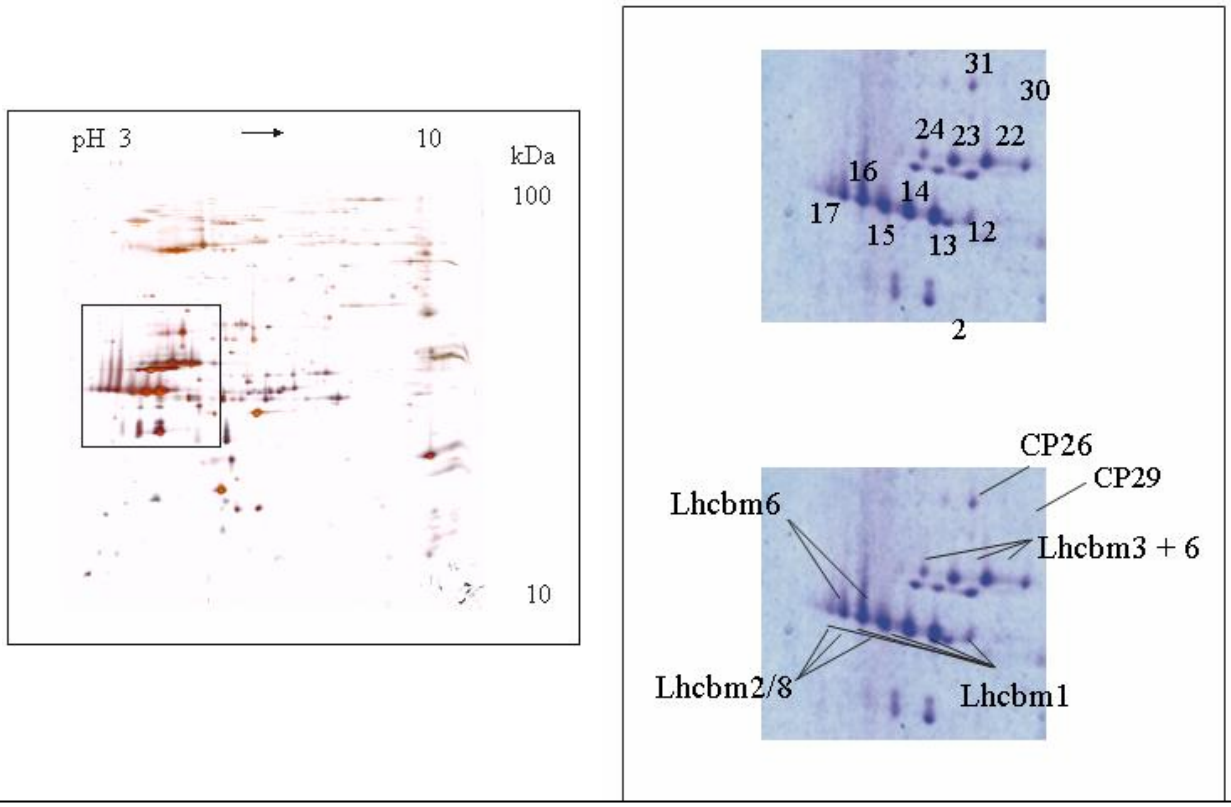


Fig 8: 2-DE protein maps of major *C. reinhardtii* LHC proteins after separation of thylakoid membranes (200 µg of protein). The upper panel shows a 2-DE map of thylakoid membranes stained with silver. The inset in the upper panel indicates the region presented in the lower panels. Each of the numbered spots on the Coomassie blue-stained 2-DE map (lower panels) was excised and digested in a gel. The subsequent MS-MS analysis resulted in the identification of LHC proteins. The labeled protein spots indicate proteins that were unambiguously identified by the MS data. Lhcbm3 + 6, Lhcbm3 and Lhcbm6; Lhcbm2/8, Lhcbm2 or Lhcbm8.

Lhcbm6 protein (Lhcb1 or CabII-1) in the upper and lower rows of spots confirms previous MS results (Hippler et al., 2001). Of note is the finding that a peptide-specific antibody directed against the N-terminal part of Lhcbm6 recognises only the upper row of spots. For the lower row of spots, Lhcbm1-specific peptides can be distinguished in spots 12 to 17, Lhcbm2- or Lhcbm8-specific ones can be seen in spots 14 to 17, and an Lhcbm6-specific one can be seen in spots 16 and 17. Thus, spots 12 and 13 can be correlated with Lhcbm1. We also specifically searched for N-terminal peptides (see Materials and Methods). From spots 14 to 16, we identified for Lhcbm2 or Lhcbm8 an

N-terminal peptide (IEWYGDPR) whose sequence matches perfectly the N-terminal amino acid sequence deduced from Edman amino acid sequencing (Hippler et al., 2000). From spot 23, we identified for Lhcbm3 an N-terminal peptide (SSGIEFYGPNR). An overlapping peptide [(K)QAPASSGIEFYGPNR] was also determined for Lhcbm3 (Table 32), indicating that two N termini exist for Lhcbm3, as suggested for Lhcbm6 (see below). Interestingly, another N-terminal peptide of Lhcbm3 (PASSGIEFYGPNR) was identified by analysis of a tryptic digest derived from LHC proteins separated by native gel electrophoresis (see below). Additionally, these data show that mature Lhcbm3 does not start with the N-terminal amino acids IEF, as does Lhcbm2 or Lhcbm8. A corresponding peptide [(K)SSGVEFYGPNR] of Lhcbm4 or Lhcbm6 was also recognised from spots 22 to 24. However, in this instance, it is possible that the peptide originated from a tryptic cut. We also identified two *lhca* gene products, Lhca3 (p14.1) and Lhca7 (p15), in spots 12 and 13. The numbers in parentheses indicate the relative mobilities of the respective Lhca proteins in SDS-PAGE, in accordance with the nomenclature of Bassi et al. (Bassi et al., 1992). The *lhca7* gene product is defined by an assembled EST contig (Grossman et al., unpublished) and has been identified for the first time at the protein level (Stauber et al., 2003).

1.4.) Identification of further Lhcbm peptides from tryptic digests of Deriphat-PAGE bands

In this analysis, we identified several peptides already found in the 2-DE analysis as well as three Lhcbm5-specific peptides and two Lhcbm4- or Lhcbm9-specific peptides (two triply charged ions with correlation factors of 5.2 and 3.8, respectively: AKWLGPYSENSTPAYLTGEFPGDYGWDTAGLSADPETF and WLGPYSENSTPAYLTGEFPGDYGWDTAGLSADPETFKR). The latter finding indicates that

Lhcbm4 or Lhcbm9 is expressed at the protein level; thus, another Lhcbm protein is added to the total number of Lhcb proteins found in this study. These large peptides were not found in the 2-DE analysis, probably because of their size. Thus, eight distinct Lhcb proteins were identified.

Chlamydomonas LHC proteins	Related gene in vascular plants	Method(s) of identification	Annotation or EST contig[a]	Position of identity to sequences in respective scaffolds of genomic database (version 1.0 assembly)
Lhcbm1	lhcII-4[b]	2-DE-LC	AB051210 AB051206 AF495473	1839
Lhcbm2	lhcII-3[b]	(Mature sequence identical to Lhcbm8)	AB051209 AB051205	385 (transit peptide sequences are found in scaffold 87)
Lhcbm3	lhcII-1.3[b]	2-DE-LC	AB051208 AB051204	354
Lhcbm4[c]	lhcb2[b]	Native PAGE-LC	AF104630	688[d]
Lhcbm5	lhcb3[b]	2-DE-LC	AF104631	168
Lhcbm6	cabII-1[b]	2-DE-LC	AF95472 M24072.1	1191 (Not complete)
Lhcbm7	lhcb2.2	(Not unequivocally)	AF479779	Not found
Lhcbm8	cabII-2[b]	2-DE-LC	AF330793	385
Lhcbm9[c]	lhcb2.2	Native PAGE-LC	AF479778	688
Lhcbm10	lhcb1.5	(Not unequivocally)	AF479777	Not found
Lhcb5 (CP26)	lhcb5	2-DE	AB050007	800
Lhcb4 (CP29)	lhcb4.3	2-DE	20021010.7231.1	538 (first 37 N-terminal amino acids not found)

[a] *C. reinhardtii* EST contig or EMBL-EBI accession numbers of previously reported genes for Lhcbm1 (AB051210, AB051206, and AF495473 [11, 42]), Lhcbm2 (AB051209 and AB051205 [42]), Lhcbm3 (AB051208 and AB051204 [42]), lhcbm4 (AF104630 [O'Connor et al., 1999]); Lhcbm5 (AF104631 [O'Connor et al., 1999]), Lhcbm6 (M24072.1 and AF495472 [11, 23]), Lhcbm7 (AF479779 [11]), Lhcbm8 (AF330793. [38]), Lhcbm9 (AF479778 [11]), Lhcbm10 (AF479777 [11]), Lhcb5 (CP26 and AB050007 [32])

[b] Homology to this vascular plant *Lhcb* gene was reported by (Elrad et al., 2002).

[c] Peptides identical to those deduced from *lhcbm4* and *lhcbm9* gene products were found.

[d] Ala207 is replaced by Arg in the genomic sequence.

Table 32: *C. reinhardtii lhca* and *lhcb* genes and gene products as determined by EST, cDNA, or genomic sequences in comparison with tryptic peptide sequences obtained by MS

1.5.) Differential N terminal processing of Lhcbm6

Interestingly, *lhcbm6*-related gene products with different molecular masses and different isoelectric points were found. The MS-MS data identified peptide WAMLGALGCQTPELLAK, which is specific for Lhcbm6, in spots 22 and 23 as well as in spots 16 and 17 with a high level of significance (Fig. 9). An independent MS analysis of 2-DE separated Lhcbm6 had already indicated that this LHCII protein might contain two alternative N-terminal protein cleavage sites (Hippler et al., 2001).

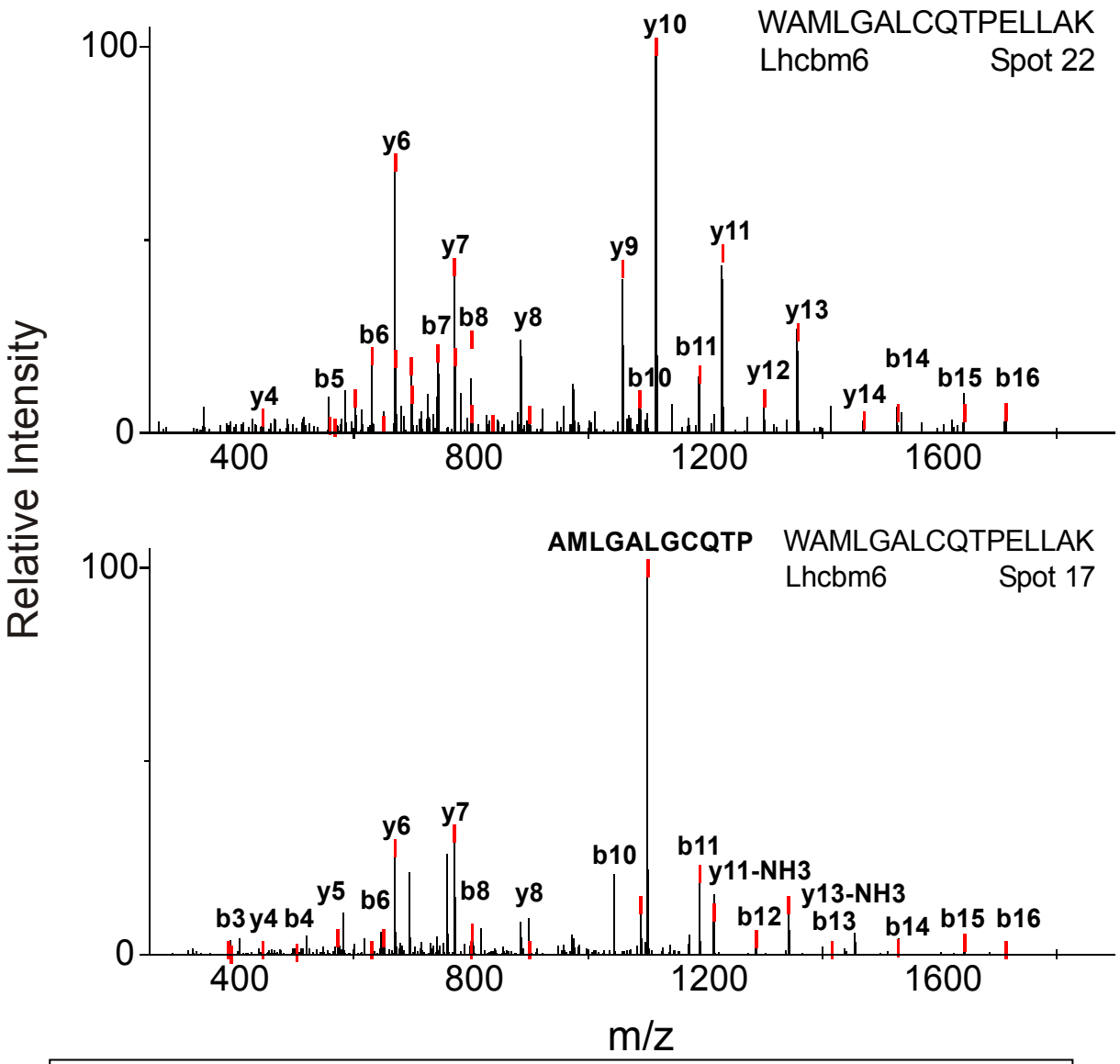


Fig. 9: Two differentially N-terminally processed forms of Lhcbm6. (A) MS-MS spectra of peptide WAMLGALGCQTPELLAK from spot 22 (without Fe) and spot 17 (with Fe). The CID mass spectra of the doubly protonated precursor ion peaks measured at *m/z* 930.5 and 930.4 for spots 22 and 17, respectively, are shown.

An HA tag was introduced into the *lhcbm6* gene in such a way that it was localised behind the second suggested N-terminal processing site [$_{28}$APKS(HA tag)SGVE$_{35}$] in the expressed gene product (Imbault et al., 1988). Anti-HA monoclonal antibodies recognised two protein bands with different molecular masses (mass difference, 1 to 2 kDa) after SDS-PAGE fractionation of thylakoid membranes from the Lhcbm6-HA-tagged algal strain. Immunoblot experiments with 2-DE separated Lhcbm6-HA-tagged thylakoid membranes, anti-Lhcbm6 peptide antibodies (directed

against a sequence upstream of the second processing site), and anti-HA antibodies revealed that the lower-molecular-mass spots recognised by the anti-HA antibodies are not recognised by the anti-Lhcbm6 antibodies; these data demonstrate that processing occurs at the N terminus (Fig. 10). Thus, the Lhcbm 2-DE mapping results and the tagging experiment independently confirmed that two differentially N-terminally processed Lhcbm6 forms exist in vivo. It should be noted that the lower-molecular-mass protein spots detected by the anti-HA antibodies are considerably shifted in their isoelectric points toward more acidic values compared to the higher-molecular-mass HA-tagged protein spots. This finding is also indicative of N-terminal protein processing, since the N-terminal part of the Lhcbm6 protein is enriched with positively charged amino acids. Fractionation of whole HA-tagged cells by onedimensional SDS-PAGE and subsequent immunoblot analyses with anti-HA antibodies revealed two protein bands with different molecular masses, indicating that N-terminal processing also occurs in whole cells and is not an artifact due to the membrane preparation.

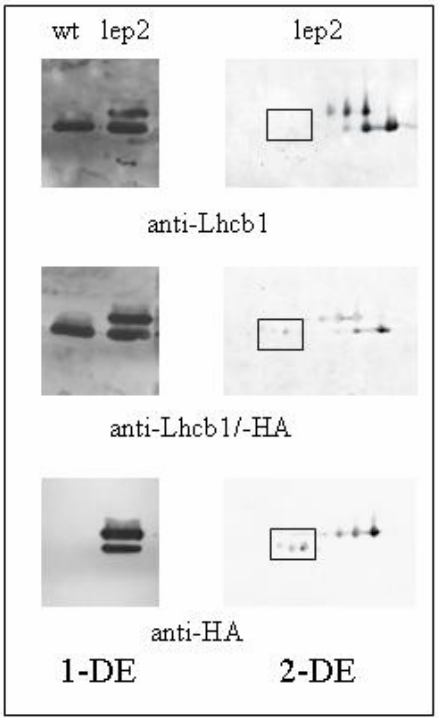


Fig. 10: SDS-PAGE separated thylakoids of wildtype and lep2 (1-DE, left panels) and 2-DE separated thylakoids of lep2 (2-DE, right panels) decorated with anti-Lhcb1, anti-Lhcb1/HA and anti-HA-antibody. Boxed areas indicate the positions of the processed forms of HA-tagged Lhcbm6.

1.6.) Phosphorylation of Lhcbm3

Currently, it is not known for *Chlamydomonas* which of the major Lhcbm proteins is phosphorylated, although it is known that these proteins become phosphorylated during the process of state transitions (Wollman and Delepelaire, 1984). In order to identify phosphorylated Lhcbm proteins, thylakoid membranes were in vitro phosphorylated and immediately subjected to native green gel electrophoresis. The green monomeric LHC band was excised, proteins were digested in

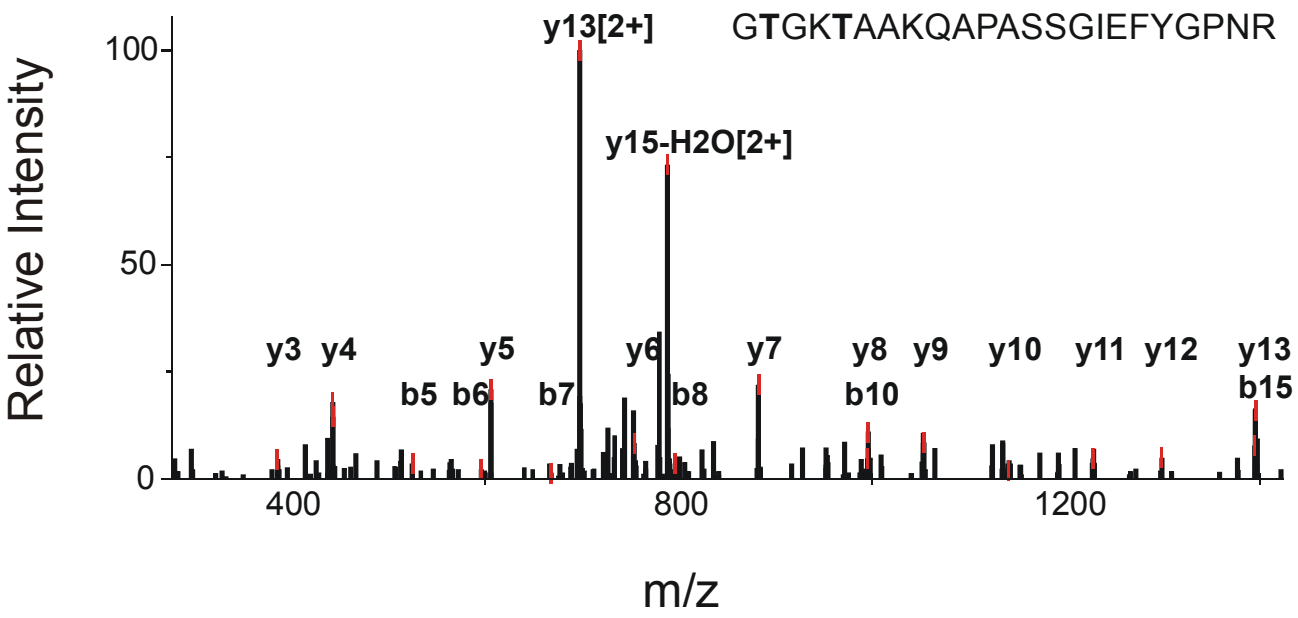


Fig. 11: MS-MS spectrum of phosphorylated Lhcbm3 peptide GTGKTAAKQAPASSGIEFYGPNR. The data indicate that either the first or the second Thr (in bold type) becomes phosphorylated. The CID mass spectrum of the triply protonated precursor ion peak measured at *m/z* 797.65 was identified from a tryptic digest of a green LHC band obtained after fractionation of in vitro-phosphorylated thylakoid membranes by native gel electrophoresis. The b-type ions b5, b6, b7, b8, and b10 represent phophorylated ions, indicating phosphorylation of either of the two Thr residues.

the gel with trypsin, and peptides were analysed by MS-MS. The MS-MS data were specifically matched with the Lhcbm database that contains a set of different N termini (see Materials and Methods, 14.7.). Thus, the Lhcbm3 peptide [(K)GTGKTAAKQAPASSGIEFYGPNR] was reproducibly found to exhibit a molecular mass shift of 80 Da, representative of phosphorylation at

a Thr or Ser residue. The peptide was found as a triply charged ion. Sequest identifies this peptide with a correlation factor of 3.2 or 3.3 when the second or first N-terminal Thr residue becomes phosphorylated, respectively, whereas no significant scores for phosphorylated Ser residues are found. Our data do not allow us to determine which of the two Thr residues is phosphorylated. We also cannot exclude the possibility that this peptide represents a mixed population, where either one of the two Thr residues is phosphorylated. A closer analysis of the fragmentation spectrum (Fig. 11) revealed a series of y- and b-type ions, including those that exhibit a shift in molecular mass of 80 Da; these data indicate that the fragmentation spectrum represents the CID pattern of the respective phosphorylated peptide. It is known that phoshorylated peptides do not fragment well in MS-MS analyses; therefore, we interpret the Sequest results as being significant, although the correlation factor is slightly under the cutoff value used throughout this study.

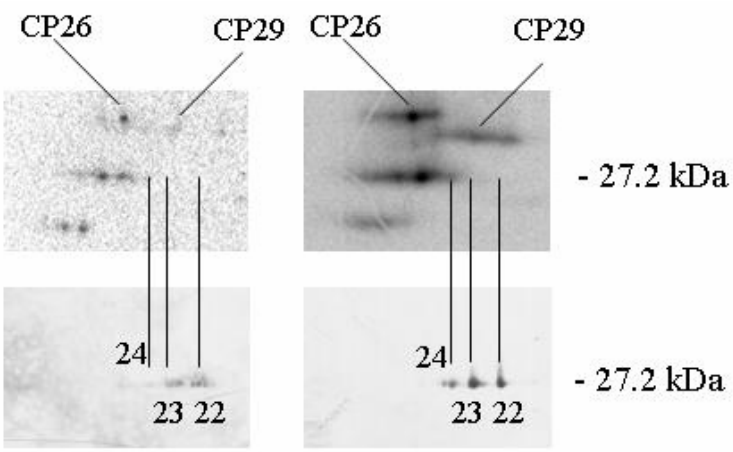
1.7.) N-terminal processing of Lhcbm3

We also detected a singly charged ion with a molecular mass of 1,394.7 Da (deviation from theoretical mass, 0.2 Da). Sequest analysis of the MS-MS data revealed a significant match, at a correlation factor of 2.00, with an Lhcbm3-specific peptide ($_{33}$PASSGIEFYGPNR$_{45}$). This peptide represents another putative N terminus for Lhcmb3 (see above). It should be noted that the phosphorylation site determined would be removed by this protein processing. Interestingly, such N-terminal protein processing can be also described for Lhcbm6 (see above).

1.8.) 2-DE Map of *in vitro* phosphorylated thylakoid membranes

To identify potentially phosphorylated proteins thylakoi
^{32}P]ATP and subsequently separated by 2-DE (Fig. 12). Th
transition mutant stm3 (not shown) and the Lhcbm6-H
Numbers relate to spots recognised by the Lhcbm6-anti
proteins with a more acidic IEP than the spots recognised
N-terminal processed forms of Lhcbm3 and Lhcbm6 appe
spots seen on the screen at this molecular weight possess
compared to the one of the N-terminal processed forms. Besides phosphorylation products of the minor LHCII proteins CP26 and CP29 could be identified.

Fig. 12: 2-DE-separation of [γ-^{32}P] ATP incubated thylakoid membranes of wildtype (upper panel, left) and lep2 (upper panel, right) and immunodetection of Lhcbm6 in 2-DE separated thylakoids of wildtype (lower panels). For orientation of the phosphorylated spots the left hand immunoblot (lower panel, left) was done on the same membrane which was exposed to the screen (upper panel, left).



CP29 is located between the PSII core and the LHCII complex and its phosphorylation has been reported to occur in photoinhibitory conditions through activation by overreduced plastoquinon (Bergantino et al., 1995). This process happens in both C3 and C4 plants, possibly as part of a redox signalling pathway between plastoquinone and the transcription machinery (Bergantino et al., 1998). Phosphorylation can modulate the spectral properties of CP29 as part of a proposed mechanism to regulate the distribution of excitation energy (Croce et al., 1996).

1.9.) 2-DE analysis of thylakoid membranes isolated from a PSI-deficient mutant

The potential to separate thylakoid membrane proteins by 2-DE opens up the possibility of starting a differential analysis of membranes from mutant and wild-type strains. One advantage of *C. reinhardtii* as a phototrophic model organism is the availability of mutant strains that are affected in photosynthesis. Thylakoid membranes isolated from a PSI-deficient mutant, a Δ*ycf4* strain, and a

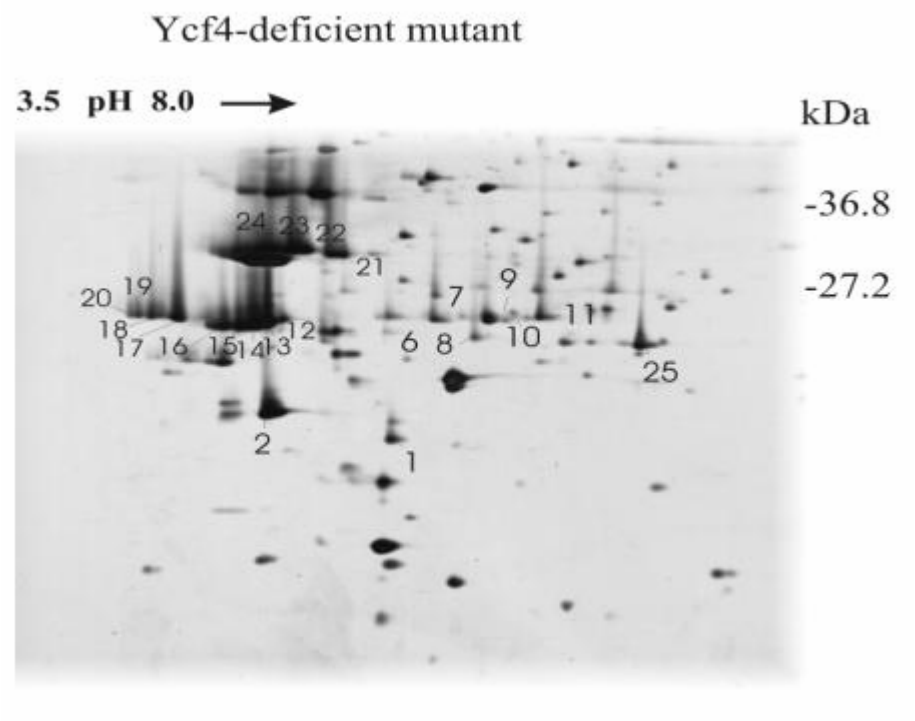


Fig. 13: 2-DE analysis of thylakoid membranes (200 µg of protein) isolated from the PSI-deficient mutant Δ*ycf4* (Boudreau et al. 1997) The 2-DE map was stained with silver.

crd1 mutant strain have been analysed by 2-DE (Fig. 13 and 14, respectively). The Δ*ycf4* strain is a knock-out mutant lacking the chloroplast encoded *ycf4* gene, which results in deficiency of PSI (Boudreau et al., 1997). The *crd1* mutant is conditionally reduced in PSI and LHCI under copper-deficiency (Moseley et al., 2000). The 2-DE maps are shown from a pH range of 3.5 - 8 and a molecular mass range from about 45 to 10 kDa to focus on LHC proteins (Figures 13 and 14). A comparison between the two maps indicates clear differences. LHCII spots 12-24 can be identified in both 2-DE separations. However , LHCI spots are strongly diminished (spots 2, 7, 8) or

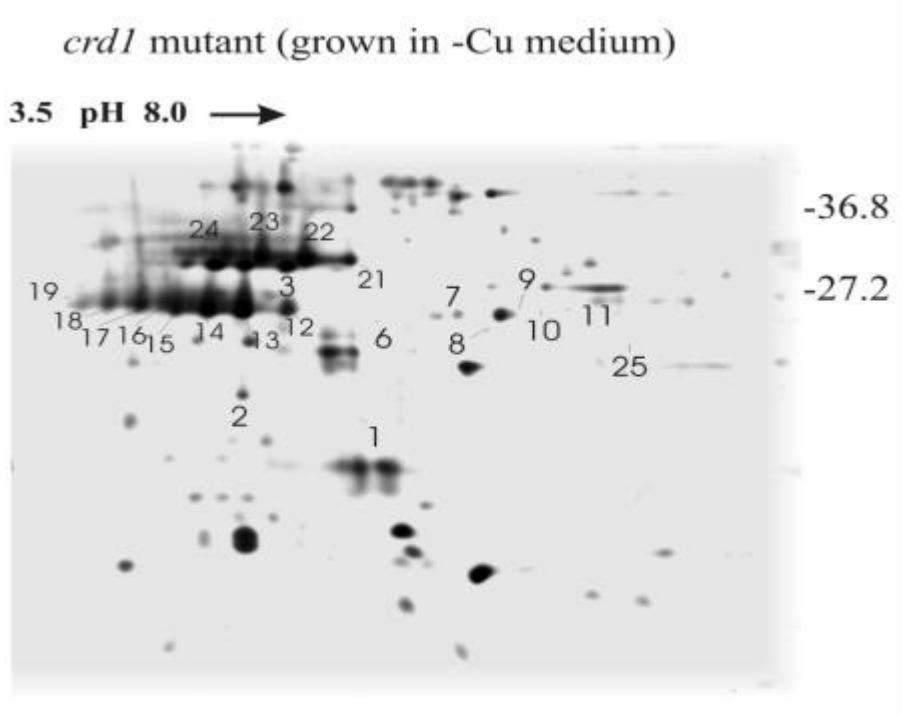


Fig. 14: 2-DE analysis of thylakoid membranes (200 µg of protein) isolated from the *crd1* mutant (Moseley et al., 2000) grown under copper-deficiency. The 2-DE maps were stained with silver.

absent (spots 1, 3, 6, 9, 10, 11, 25) in the 2-DE map of thylakoids isolated from copper-deficient *crd1* cells, whereas these spots are present in the map of thylakoids isolated from the PSI-deficient mutant. In 2-DE maps from *crd1* thylakoids isolated from copper-supplemented cells or from thylakoids isolated from copper-deficient Δ*ycf4* cells, all LHCI spots are present (data not shown). It has been observed previously that strains carrying deletion mutations in the core polypeptides of PSI (Girard-Bascou et al., 1987; Takahashi et al., 1991) still contain LHCI proteins. Our 2-DE results are compatible with this finding, in addition our results reveal the presence of spots 1, 2, 6, 7, 8, 9, 10, 11 and 25 (see numbering; Figures 13 and 14), thus proving that these LHC proteins accumulate with the thylakoid membranes independently from the PSI core complex. These results also show that the positions of LHCI spots on the 2-DE maps are independent of the presence of the PSI complex and hence are not due to incomplete solubilisation of PSI complexes. Immunoblotting results indicated the absence of LHCI subunits when *crd1* mutant cells were grown in copper-deficient medium (Moseley et al., 2000). The 2-DE results (Figure 14) demonstrate that the *crd1* mutation strongly affects all LHCI spots so far identified with our gel system, which strengthens the

view that the *crd1* mutation affects the entire LHCI proteins under copper-deficiency. It is of note that other spots of unknown identity are absent or enriched in the 2-DE map of copper-deficient *crd1* thylakoids compared with that of copper-supplemented Δ*ycf4* thylakoids.

1.10.) 2-DE Analysis of the state transition mutant stm3

The stm3 mutant is a randomly tagged *ble*-gene transformant that was screened for by fluorescence video imaging (Kruse et al., 1999). Non-functional state transitions could be directly related to the suppression of a gene encoding for an RNA-binding protein that leads to reduction in a single LHCII type 1 protein (Mussgnug, 2004). The phenotype of non-functional state transitions is reflected in a defect in the lateral migration process of LHCII proteins and no docking to photosystem I complexes. Nevertheless state 2 induced phosphorylation of LHC II proteins could be shown (Mussgnug, 2004). Differences in the protein pattern between wildtype and stm3 could be identified by Western blotting of 2-DE separated thylakoid membranes (Fig. 15). Immunoblots decorated with a specific anti-Lhcb1-antibody showed the non-expression of the Lhcbm6-protein. This protein spot was present in the reconstituted mutant nc1, that was made from stm3 by transformation with the *Chlamydomonas reinhardtii* gene nab1, coding for a putative nucleic acid binding protein (Acc. No.: AY157846).

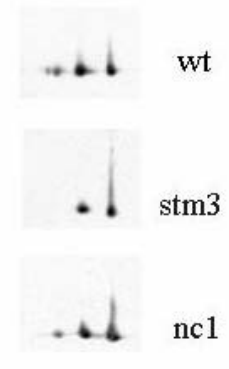


Fig. 15: Western Blots of 2-DE separated thylakoids from wildtype and the stm3 and nc1–mutants, decorated with a specific anti-Lhcb1-antibody.

1.11.) 2-DE analysis of the npq mutant npq5

Mutant npq5 was created by random insertion of the *ble*-gene into wildtype *C. reinhardtii*. The mutant showed reduction in non-photochemical quenching and the deleted gene was identified as one coding for the Lhcbm1 protein. (Elrad et al., 2002) Applying 2-DE analysis of thylakoids isolated from npq5, isoforms deduced from this gene could be shown to be deficient in this mutant both by silver staining and immunoblotting with the P25K antibody (Fig. 16). The spots absent in npq5 have been shown to contain the Lhcbm1 protein by MS in independent experiments (Table 32).

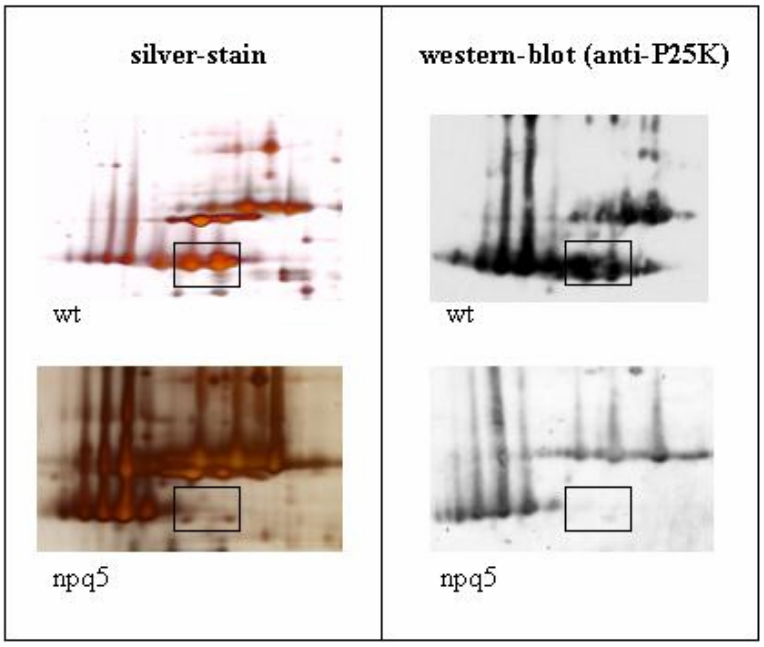


Fig. 16: 2-DE analysis of thylakoids from wildtype and the npq-mutant npq5. The left panel shows silver-stainings and the right panel western blots decorated with anti-p25K-antibodies.
Boxed regions indicate position of lhcbm1 proteins deleted in npq5.

2.) Using the PsaF-deficient mutant 3bF to screen for mutants that are deficient in assembly of light harvesting proteins

The PsaF deletion mutant 3bF was created from wildtype *Chlamydomonas reinhardtii* in order to study the importance of the PSI subunit PsaF in electron transfer from PSII to PSI (Farah et al., 1995). This mutant is sensitive to high light irradiation since oxidative stress evoked by the disruption of fast and efficient electron transfer between the photosystems causes generation of oxygen radicals and eventually toxic effects in the thylakoids. It was postulated that a PsaF-deleted mutant could survive high light stress by reducing number and size of their antenna. Therefore mutant 3bF seemed to be the ideal background for isolating mutants that are affected in the assembly of light harvesting proteins. 3bF was the subject to a high light resistance screen for mutants created by random insertional mutagenesis with the *ble*-gene.

2.1.) Mutant screen

The PsaF-deficient *Chlamydomonas reinhardtii* strain 3bF was transformed with plasmid psp115 containing the *ble*-gene which confers resistance to the antibioticum zeocin (Stevens et al., 1996). Random insertion of the plasmid resulted in 22 zeocin-resistant colonies that survived on zeocin-containing TAP plates under normal light conditions. Upon transfer to minimal salt medium plates under high-light conditions (300 µE) only one of these mutant strains (S2) survived. The wildtype strain also survived high-light conditions while the PsaF-deficient strain 3bF died. (Fig. 17) 3bF was shown to survive in low light conditions (Hippler et al., 2000). Chl-determination by HPLC-

analysis and photospectrometry revealed Chl b-deficiency in thylakoids and whole cells of S2 (Figures not shown).

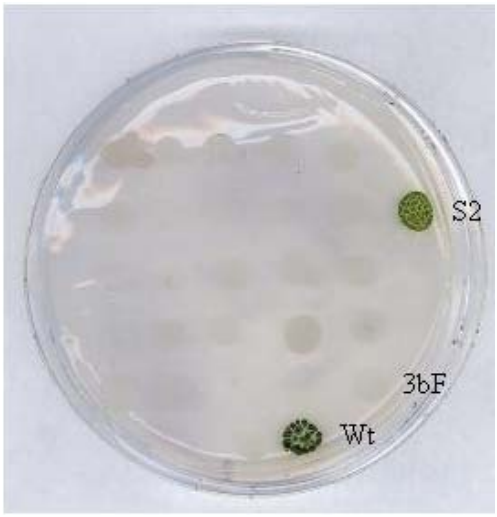


Fig. 17: Mutant screen for high-light resistant mutants. The strain 3bF was mutagenised by the glass beads method with plasmid psp115 which carries the ble-gen, confering resistance to the antibioticum zeocin. The zeocin-resistant mutants were transfered to HSM-medium under high-light conditions.

2.2.) Characterisation of mutant strain S2

Chl-determination revealed Chl b-deficiency in thylakoids and whole cells of this strain. Western blot analysis of SDS-PAGE (Laemmli, 1970) separated thylakoids from S2, 3bF, wildtype and mutant cbs3 (see below) confirmed that S2 is PsaF-deficient like its original strain 3bF, whereas wildtype and cbs3 contain PsaF (Fig. 18). Incubation of the Western blots with anti-PsaD as a loading control proved that thylakoids of the four strains contain equal amounts of PSI. The Chl b-deficient mutant cbs3 was included into the analysis to compare the phenotypes of two Chl b-deficient mutants (Table 34) whose Chl b-deficiency have most probably different reasons. In contrast to S2 cbs3 has already been genetically analysed. Cbs3 is a Chl b-deficient mutant with a deletion in the CAO gene coding for chlorophyll a oxygenase by insertional mutagenesis (Tanaka et al., 1998). The mutation in S2 has been stable for three years, and is expressed by a high Chl a/Chl b ratio (Table 34), yellowish-green colour, high-light resistance and resistance against the antibioticum zeocin. In contrast the Chl b-deficient high light resistant mutant 2B which was

created by directed spontaneous mutation (Hippler et al., 2000) has lost its phenotype during the course of this study and was subject to suppression of the Chl b-deficiency which resulted in high-light sensitivity.

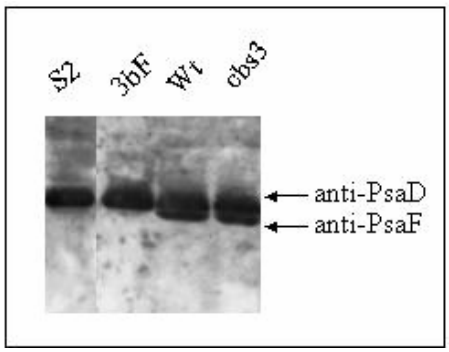


Fig. 18: Western blot of thylakoids from S2, 3bF, wt and cbs3, incubated with anti-PsaF and anti-PsaD.

2.2.1) 2-DE analysis of mutant strain S2

Silver staining of 2-DE separated thylakoids of S2 revealed reduced intensity of a protein spot which was identified by mass spectrometry to contain the LHCII proteins Lhcbm1 and Lhcbm2/8 (Fig. 19). Western blot experiments with the antibody P25K confirmed this result. Similar experiments with the mutant cbs3 showed no difference to wildtype (results not shown).

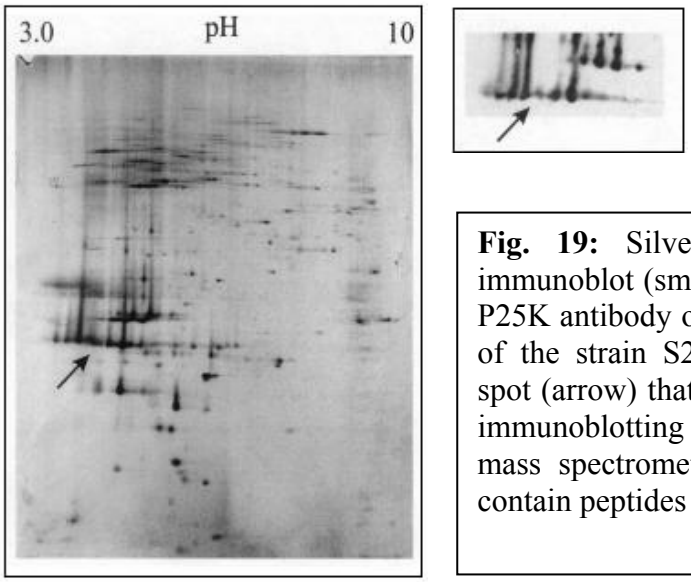


Fig. 19: Silverstaining (big panel) and immunoblot (small panel) incubated with the P25K antibody of 2-DE-separated thylakoids of the strain S2 revealed reduction in one spot (arrow) that was attributed to LHCII by immunoblotting and mass spectrometry. By mass spectrometry this spot was found to contain peptides of Lhcbm 2/8 and Lhcbm 1.

2.2.2) 77K fluorescence analysis

To have a first insight into effects of interconnection between light harvesting antennae and the photosynthetic reaction centers, we measured 77 K fluorescence emission spectra for wildtype and the mutant strains. The fluorescence emission spectrum of the emitting pigment proteins at low

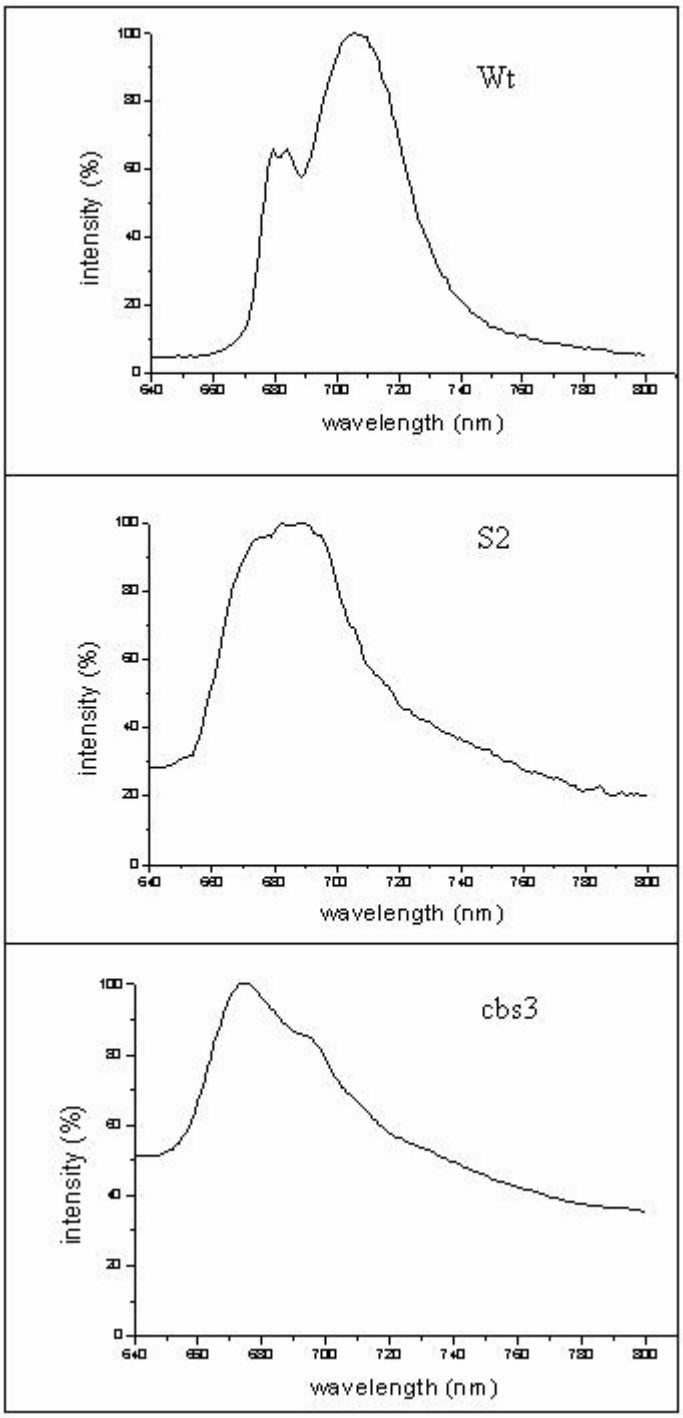
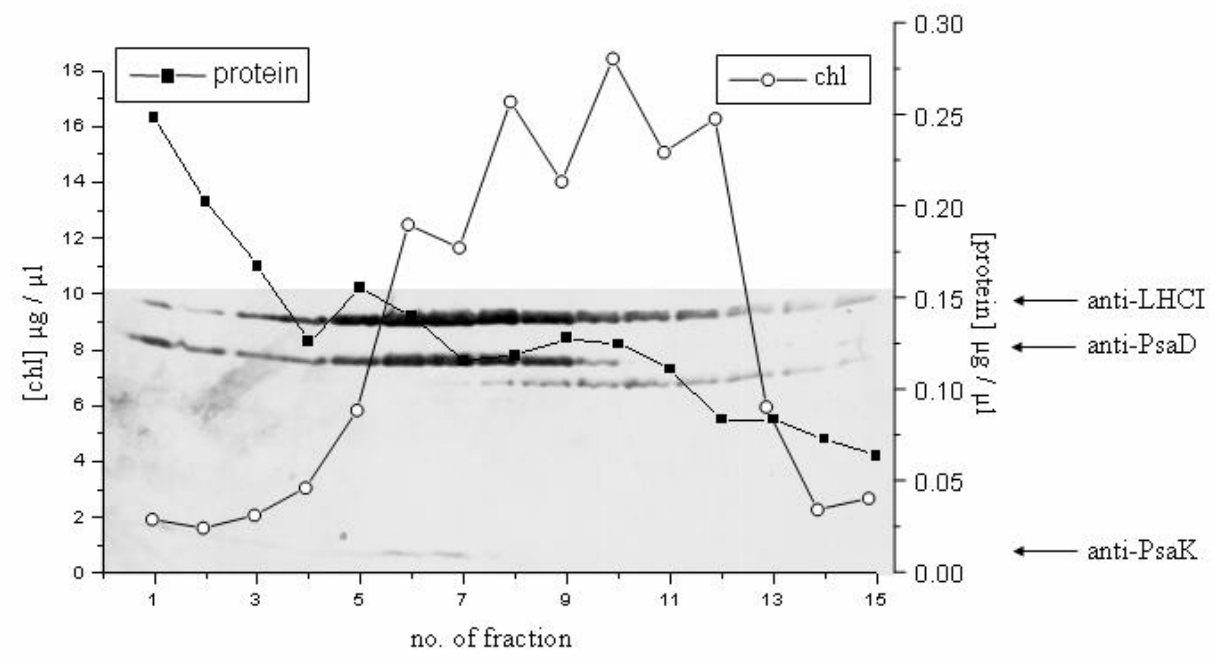


Fig. 20: 77 K fluorescence emission spectra for wildtype and the mutant strains S2 and cbs3.

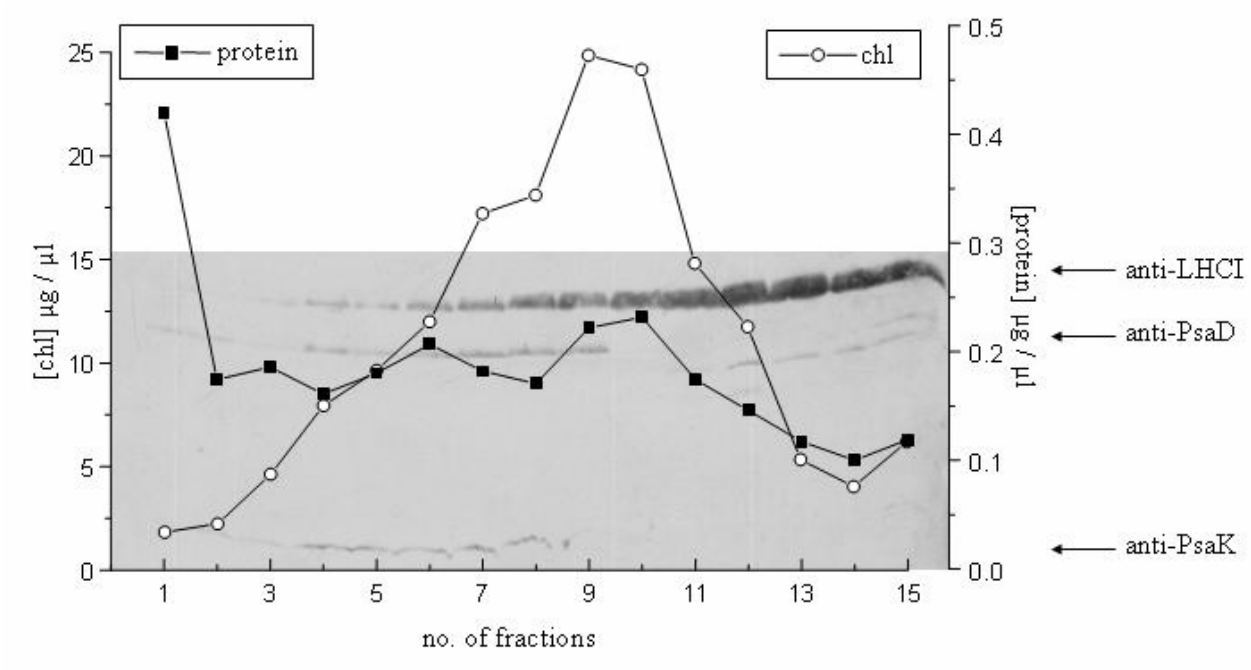
temperature depends on the physical environment and functional interaction of these proteins with other pigment-binding proteins. Fluorescence peaks at 685 nm and 709 nm have been found to be characteristic for LHCII attached to PSII and LHCI attached to PSI, respectively, whereas isolated LHCI antennae show a fluorescence maximum at 705 nm (Wollman and Bennoun, 1982). For thylakoid membranes prepared from wildtype peaks were found at 685 and 709 nm, which are in accordance to literature (Fig. 20). In contrast for the mutant cbs3 a peak was found at 674 nm and a shoulder at 696 nm, and for the mutant S2 a broad undefined emission from about 685 to 700 nm could be observed.

2.2.3) Western blots of fractionated thylakoid membranes

a) wildtype



b) S2



c) cbs3

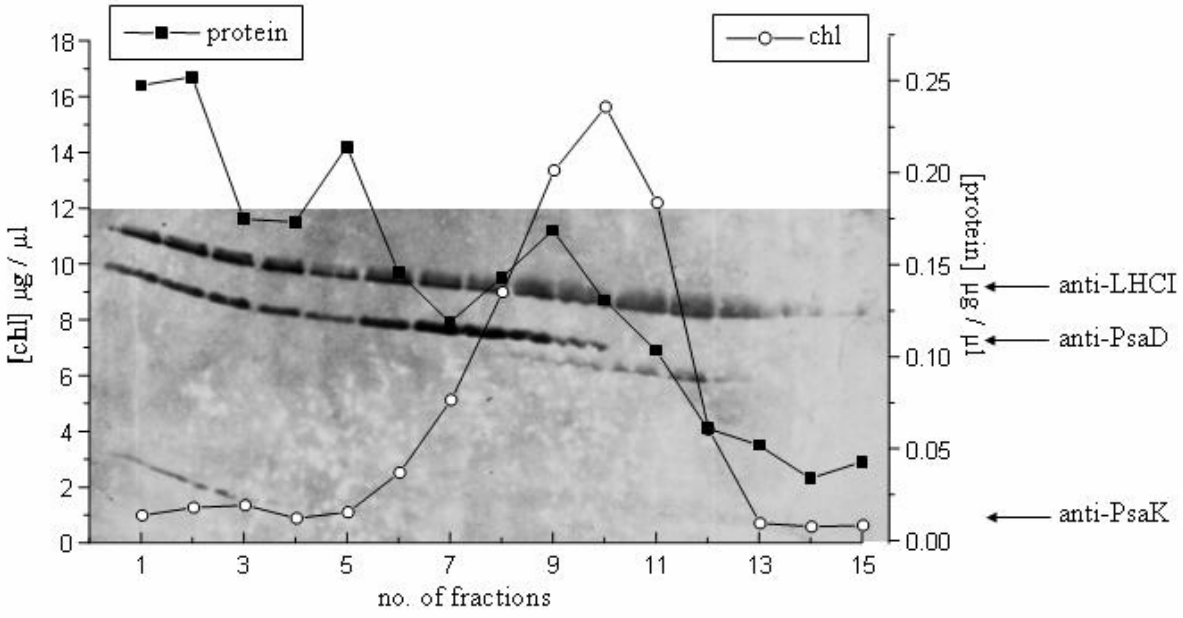


Fig. 21: Western blots of SDS-PAGE separated thylakoid fractions. Thylakoids from a) wildtype, b) S2 and c) cbs3 were solubilised with β-dodecylmaltoside and fractionated by density gradient ultracentrifugation in a continuous sucrose gradient. Western blots were incubated with anibodies against PsaD, Lhca3 (anti-LHCI) and PsaK. (40 µl of each fraction were loaded).

Isolated thylakoids (Chua and Bennoun, 1975) from wildtype and the mutants S2 and cbs3 were solubilised with β-dodecylmaltoside and fractionated on a continuous sucrose gradient by density gradient ultracentrifugation. For each strain 15 fractions of 1 ml were collected. 40 µl of each fraction were separated by onedimensional SDS-PAGE (Laemmli, 1970) and immunoblotted with anti-14.1-, anti-PsaD- and anti-PsaK-antibodies (Fig. 21). The Western blots revealed that LHCI proteins do not co-segregate as consistently with PSI in the mutants as found in wildtype. In wildtype the PSI : LHCI ratio is roughly the same in fractions 3 - 15, with a peak in overall content of PSI and LHCI in fractions 5 - 9 (Fig. 21a). For S2 the immunoblots show that fractions 8 - 15 are relatively enriched for LHCI proteins (Fig. 21b). In cbs3 fractions 6 - 9 contain significantly less LHCI than PSI, fractions 10 - 13 are more abundant in LHCI, whereas fractions 14 - 15 contain only LHCI (Fig. 21c). Fractions that were enriched in PSI from each strain were separated by SDS-PAGE and immunoblotted as above (Fig. 22). The Western blots showed that thylakoid fractions

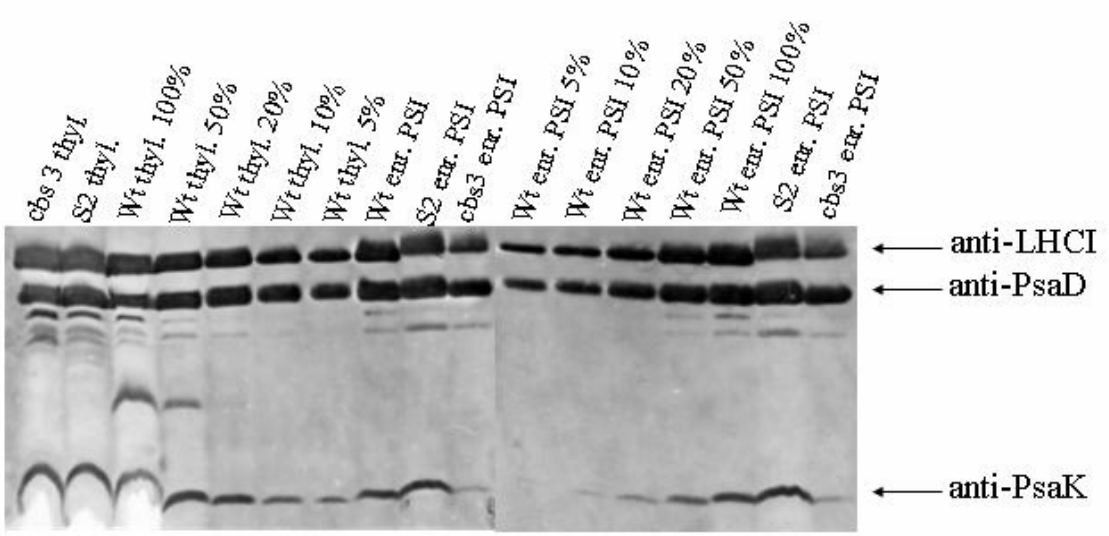


Fig. 22: Western blots of SDS-PAGE separated thylakoids and PSI-enriched thylakoid fractions. Fractions of the solubilised thylakoids from wildtype, S2 and cbs3 that showed the highest enrichment in PSI as indicated by immunoblotting with anti-PsaD were loaded on an equal protein basis (400 µg protein) together with thylakoids from each strain and a dilution series of wildtype thylakoids and enriched PSI fractions. The western blots were incubated with anti-PsaD, anti-LHCI and anti-Psak antibodies.

enriched in PSI from cbs3 and S2 are relatively redundant in LHCI, whereas for wildtype LHCI and PSI levels are almost equal. PSI-enriched fractions of cbs3 lack PsaK, while PsaK is found in equal amounts in PSI-enriched fractions of wildtype and S2 and in thylakoids from S2, cbs3 and wildtype.

2.2.4.) Western blotting of 2-DE separated (Deriphat-PAGE + SDS-PAGE) thylakoids of wildtype, S2 and cbs3

Thylakoids of wildtype, S2 and cbs3 were separated by Deriphat-PAGE (Peter et al., 1991). Green bands containing the native complexes were excised, denatured by heating at 55°C in sample buffer and applied to onedimensional SDS-PAGE (Schagger and von Jagow, 1987). The separated bands were immunoblotted and probed with antibodies against LHC and photosystems proteins. (Fig. 23)

While thylakoids of wildtype and cbs3 separated into three green bands by Native PAGE, for S2 only two bands were visible. In wildtype the PSII core light harvesting complexes CP43 and CP47 were detected only in the upper band (A) whereas for cbs3 these proteins were also found in a lower band (E). Since the PSII center core polypeptide D1 was only detected in the upper band, this leads to the interpretation that a portion of the core pigment proteins CP47 and CP43 are detached from D1 in the mutant. For the LHCII-antibody P25K recognising the LHCII-subfamily, no differences can be found between the mutants and wildtype, but the antibody specific for Lhcbm6 detected proteins in two bands, whereas for the Chl b-deficient mutants cbs3 and S2 only lower bands (D,G) were recognised. This could lead to the interpretation that LHCII-trimers are either not present in thylakoids of the mutants or not separated in the combined native and denaturing separation. The abundance of PSI was assessed using anti-PsaF-antibodies for wildtype and cbs3, and PsaD for S2. Interestingly while for wildtype and S2 PSI was exclusively detected in the upper band (A,F), for

cbs3 PsaF recognised two bands (A,E), which indicates that there are two PSI-populations of different molecular weight. This suggests that a part of PSI is detached from the LHCI-PSI complex. LHCI in wildtype was only detected in the upper band, whereas for cbs3, LHCI could be detected in the upper band and a lower band (D), which supports the interpretation of two LHCI-PSI complexes, whereas for S2 LHCI was only found in the lower band. This demonstrates that LHCI is completely detached from PSI in mutant S2.

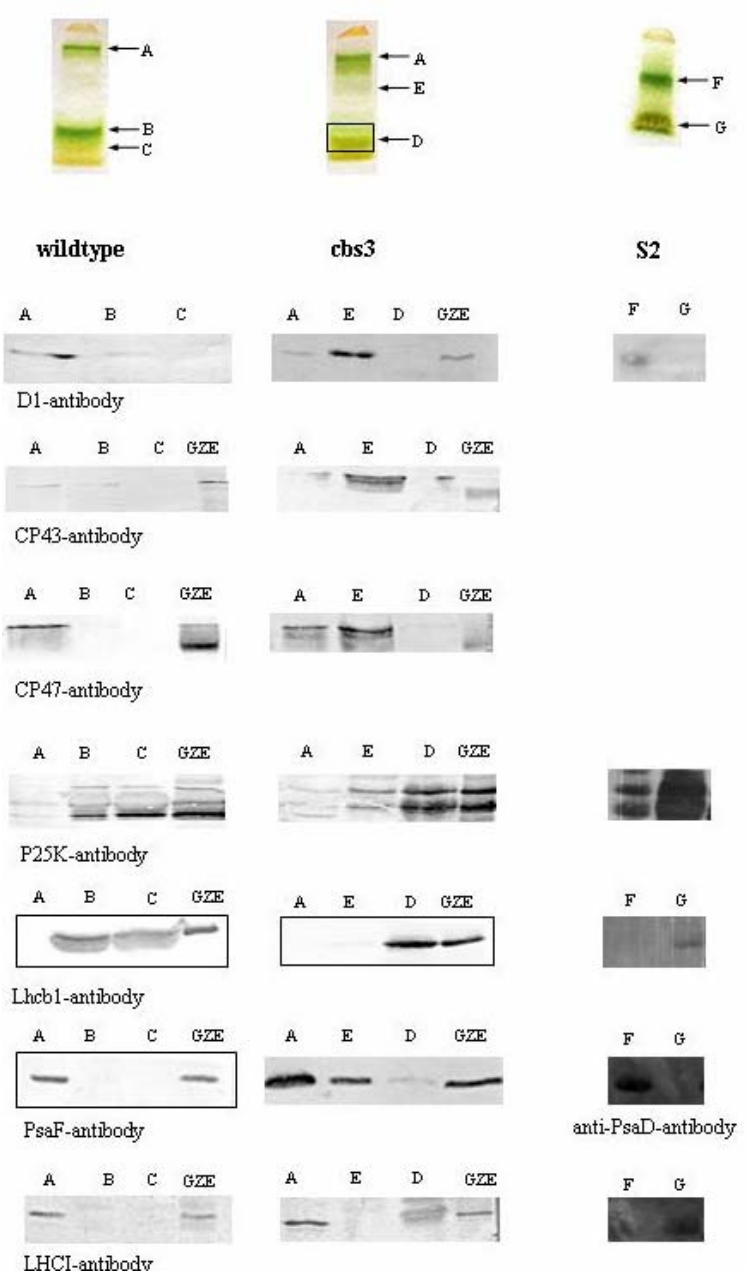


Fig. 23: Western blotting of 2-DE separated (Deriphat-PAGE + SDS-PAGE) thylakoids of wildtype, S2 and cbs3. Thylakoids were separated by Deriphat-PAGE (Peter et al., 1991). Green bands containing the native complexes were excised, denatured by heating at 55°C in sample buffer and applied to onedimensional SDS-PAGE (Schagger and von Jagow, 1987). The separated bands were immunoblotted and probed with antibodies against LHC and photosystems proteins as indicated.

2.2.5.) Oxygen uptake assay

Oxygen uptake was measured for thylakoids (Chua and Bennoun, 1975) from wildtype, S2 and cbs3 in dependence of the light intensity. In the assay oxygen evolution by PSII is inhibited by the PSII-inhibitor DCMU. The artificial e^--donor DCPIP renders electrons to PSI, which are then taken up by the artificial e^--acceptor methylviologen. In the net sum of this reaction 1 mol of O_2 is used up to produce 2 mols of H_2O_2 (Mehler, 1951). To avoid breakdown of H_2O_2 into water and oxygen by catalase, the catalase is inhibited by sodium azide. The physiological measurements revealed higher light saturation values and greater slopes for light intensity for oxygen uptake when isolated thylakoids from mutant strains S2 and cbs3 are compared with wildtype (Table 33).

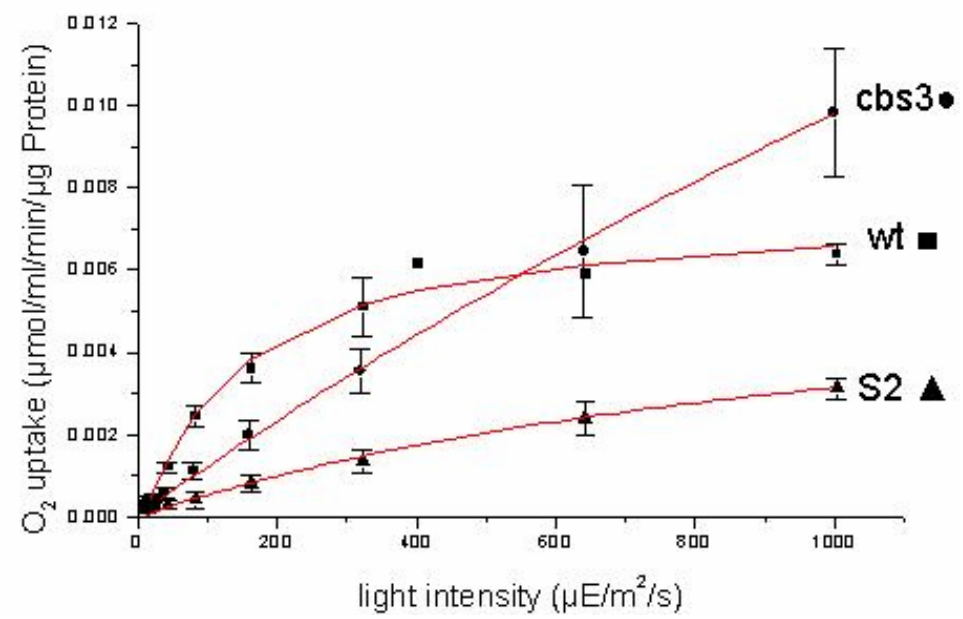


Fig. 24: Oxygen uptake assay for thylakoids from wildtype (triangles), S2 (squares) and cbs3 (circles).

	wildtype	S2	cbs3
maximal oxygen uptake (v_{max}) (µmol/ml/min/µg Protein)	0.0080 ± 0.0004	0.0068 ± 0.0013	0.0546 ± 0.0144
half-maximal saturation (k_m) (µE/m^2/s)	141 ± 21	1155 ± 355	4598 ± 1419

Table 33: Maximal oxygen uptake and half-maximal saturation value for oxygen uptake assays for thylakoids from wildtype, S2 and cbs3.

2.2.6) Pigment analysis of mutant strain S2

Thylakoids of wildtype and mutant strains 3bF and S2 grown under normal light and nutrient conditions were analysed by HPLC for pigment content. Thylakoids of S2 show significantly increased levels of total carotenoids. In particular levels of β-carotene, violaxanthin and lutein are raised between 1.5- and 2-fold, whereas neoxanthin is less abundant in the mutant. β-branch carotenoids to α-branch carotenoids ratios are higher in wildtype than in S2, as are the ratios of the combined β-branch xanthophylls neoxanthin and violaxanthin to the α-carotene-derived lutein as well as to β-carotene. Levels and ratios in 3bF are very similar to the ones seen in wildtype, except for the high N/V ratio and high β-branch xanthophylls levels. (Table 34)

	wildtype	3bF	S2	cbs3
neoxanthin (Nx)	8.3 ± 2.7	12.7 ± 0.1	6.8 ± 0.7	n.m.
violaxanthin (Vx)	10.2 ± 1.3	7.4 ± 0.8	21.4 ± 1.3	n.m.
lutein (Lu)	7.7 ± 0.9	9.7 ± 1.1	16.7 ± 2.0	n.m.
β-carotene (β-C)	7.0 ± 0.9	6.4 ± 0.8	11.3 ± 1.0	n.m.
Nx / Vx	0.81 ± 0.23	1.72 ± 0.18	0.32 ± 0.03	n.m.
(Nx + Vx) / Lu	2.47 ± 0.76	2.05 ± 0.35	1.70 ± 0.20	n.m.
(Nx + Vx + β-C) / Lu	3.37 ± 0.93	2.75 ± 0.49	2.40 ± 0.20	n.m.
(Nx + Vx) / β-C	2.67 ± 0.51	3.15 ± 0.21	2.53 ± 0.31	n.m.
Chl a	42.2 ± 2.1	39.9 ± 2.8	41.7 ± 1.1	38.6 ± 16.1
Chl b	24.4 ± 4.3	26.2 ± 4.6	0.9 ± 1.6	0
Chl (a + b)	66.6 ± 2.9	66.1 ± 3.1	41.7 ± 1.1	38.6 ± 16.1
Chl a/b	1.78 ± 0.44	1.57 ± 0.40	-	-
Car_{total} / Chl	0.50 ± 0.07	0.51 ± 0.07	1.35 ± 0.06	n.m.

Table 34: Pigment analysis of thylakoids from wildtype and mutants 3bF, S2 and cbs3. Contents for specific carotinoids (N, V, L, β-C) are given as percentage of overall pigment content (including chlorophyll). For cbs3 only chlorophyll content was measured.

Discussion

1.) High-resolution 2-dimensional gel electrophoresis of thylakoid membranes

The separation of thylakoid membrane proteins isolated from *C. reinhardtii* by twodimensional gel electrophoresis demonstrated that proteins such as the LHCPs, which span the membrane with three transmembrane domains, can be reliably separated by a high-resolution gel system. In addition the data show that this 2-DE system can be used for differential analysis of wildtype and mutant thylakoids. (Fig. 4, 5, 13-16)

To answer the question whether hydrophobic proteins such as the reaction centre proteins of PSI can be separated by using our 2-DE protocol, the presence of the PsaA polypeptide was tested for by immunoblotting. The PSI reaction centre subunit PsaA possesses 11 transmembrane domains, which leads to a GRAVY score of about 0.2. In comparison the PSII reaction centre subunit D1 has five transmembrane domains and a GRAVY score of about 0.28. When wildtype thylakoids were separated by 2-DE and analysed in an immunoblot experiment, we could visualise about four spots, when the blot was probed with anti-PsaA specific antibodies. These spots have isoelectric points ranging from about 5.8 - 6.6 and a molecular mass of about 70 kDa. They match with spots on the silverstained gel shown in Figure 4 (Fig. 4, boxed region), indicating that proteins which are more hydrophobic than the LHCPs, with up to 11 transmembrane domains can be separated by 2-DE. The

fact that (Naver et al., 2001) also succeeded in the separation of PsaA by using the same 2-DE protocol, clearly supports this finding. However, a high number of membrane proteins exist that have GRAVY scores higher than 0.5. It is not clear whether the 2-DE procedure presented here will also facilitate the separation of these proteins. Nevertheless, the reproducible 2-DE separation of transmembrane proteins such as the LHCPs was a step into functional proteomics of thylakoid membrane proteins from *C. reinhardtii*.

1.1) Separation of light harvesting proteins by 2-DE

The separation of LHCPs from PSII and PSI by 2-DE enabled differentiation between more than 30 LHCP spots. For the major LHCII proteins of *Arabidopsis* five Lhcb1, four Lhcb2 and one Lhcb3 genes have been identified, whereas only six genes encoding the LHCI antenna proteins have been found (Jansson, 1999). The 2-DE analysis of thylakoids identified at least 13 spots that correspond to major LHCII proteins (Fig. 7). These high number of spots may on one hand, also reflect the high number of genes that encode for LHCII proteins in *Chlamydomonas*, but on the other hand, can be rationalised by differential processing of the transit peptide or mature protein as shown by mass spectrometric analysis of Lhcbm6. Such a differential processing may lead to the removal of charged amino acids so that preprocessed and processed proteins differ in their IPs.

1.2.) Analysis of LHCI by 2-DE and immunoblotting

In higher plants, LHCI can be separated into three subcomplexes; the LHCI-730 complex is a heterodimer composed of Lhca1 and Lhca4 and LHCI-680A and LHCI-680B are homodimers of Lhca3 and Lhca2, respectively (Knoetzel et al., 1992; Schmid et al., 1997). This clear differentiation could not be obtained for the LHCI complex isolated from *C. reinhardtii* (Bassi et al., 1992), where the LHCI subcomplexes having a 77 K fluorescence emission of 705 or 680 nm did not differ in their subunit composition. From this 2-DE analysis it also appears that the subunit stoichiometry of the LHCI complex differs from that of higher plants, since several LHCI subunits are of rather low abundance. This heterogeneity may have functional impact and assembly of a rather heterogeneous LHCI complex could be a way to modulate transfer of excitation energy to the PSI reaction center. In *C. reinhardtii* at least six different genes encoding LHCI subunits exist (Bassi et al., 1992). The fact that 18 LHCI spots could be detected by mass spectroscopy and immunoblot analysis indicate that posttranslational modifications must account for the presence of some of these spots. The use of two peptide-specific antibodies support this interpretation, since the anti-14.1 and anti-15.1 antibodies detect four and two different spots, respectively. (Fig. 6 and 7) Different processing of the transit peptide or the mature protein, as in the case of the Lhcbm6 protein, could be one reason for multiple forms of LHC proteins. Interestingly all these LHCI spots are strongly diminished or absent from 2-DE maps of *crd1* thylakoids isolated from copper-deficient cells (Fig. 14). This implicates a regulatory mechanism for the entire LHCI complex which leads, due to the lack of *crd1* function, to a down-regulation of LHCI subunits under copper-deficiency. The separation of thylakoid membrane proteins of *C. reinhardtii* by twodimensional gel electrophoresis proved feasible, which opened the door for systematic proteomics of thylakoid membrane proteins and other protein-rich membrane systems.

1.3.) 2-dimensional maps of Lhcb proteins

The detailed twodimensional protein maps of light-harvesting proteins from *C. reinhardtii* were established by analysing excised trypsin-digested protein spots with MS taking advantage of the sequence information provided by the *Chlamydomonas* genome project, which allowed to retrieve peptide sequences from a large *Chlamydomonas* EST database as well as from a genomic database by using the MS data. Eight distinct Lhcb proteins could be predicted six of which were completely represented in the first *C. reinhardtii* genomic database assembly (version 1.0) (Table 32). For the other gene products, complete genes could not be reconstructed, but the genomic assembly did represent partial sequences.

The MS and succeeding bioinformative analyses revealed a high level of complexity of the light-harvesting proteins in *C. reinhardtii*. In addition to the large number of different LHC proteins, N-terminal protein processing of the *lhcbm3* and *lhcbm6* gene products could be identified. This finding, which was evident from the MS data as well as from the Lhcbm6-HA tagging experiment, demonstrates that two differentially N-terminally processed forms of Lhcbm3 and Lhcbm6 exist in thylakoid membranes. (Fig. 9 and 10)

1.4.) Putative processing sites of Lhcbm6

The pre-Lhcbm6 protein has a theoretical IP value of 5.98, whereas the putative mature Lhcbm6 (starting from Ala28) has one of 4.8. Spots 22 - 24, that were analysed by mass spectrometry have an experimentally determined IP of 5.4 - 5.5, which matches the theoretical IP expected when Lys9 is the first N-terminal amino acid (Compute pI/MW - Expasy homepage). Differential processing of pre-LHCPs has been reported in *in vitro* studies for wheat, pea, tobacco, tomato and corn (Clark et al., 1989; Clark et al., 1990; Cline, 1988; Kohorn and Yakir, 1990; Lamppa and Abad, 1987; Pichersky et al., 1987) when pre-LHCPs were imported into isolated chloroplasts from wheat or pea. When the putative transit peptide of Lhcbm6 from *C. reinhardtii* is compared with the processing sites of the pre-Lhcbm6 sequence from wheat (Hippler et al., 2001) it appears that the secondary processing site of the wheat sequence, which is believed to be within the mature protein (Clark and Lamppa, 1991), is very similar to the site in the *C. reinhardtii* sequence that was suggested from sequence alignments (Bassi et al., 1992). Furthermore, the primary processing site (Mullet, 1983) of the wheat transit sequence resembles the site in the *C. reinhardtii* sequence which can be deduced from the mass spectrometric results (Figure 25). Interestingly, in an organelle-free assay enriched for the chloroplast-soluble processing enzyme (Abad *et al*., 1989) as well as with the recombinant stromal processing peptidase (Richter and Lamppa, 1998), pre-Lhcbm6 is processed at the second site. However, Thr-3 starting the N-terminus at the primary processing site is found to be phosphorylated in thylakoid membranes from spinach and *Arabidopsis* (Michel et al., 1991; Vener et al., 2001).

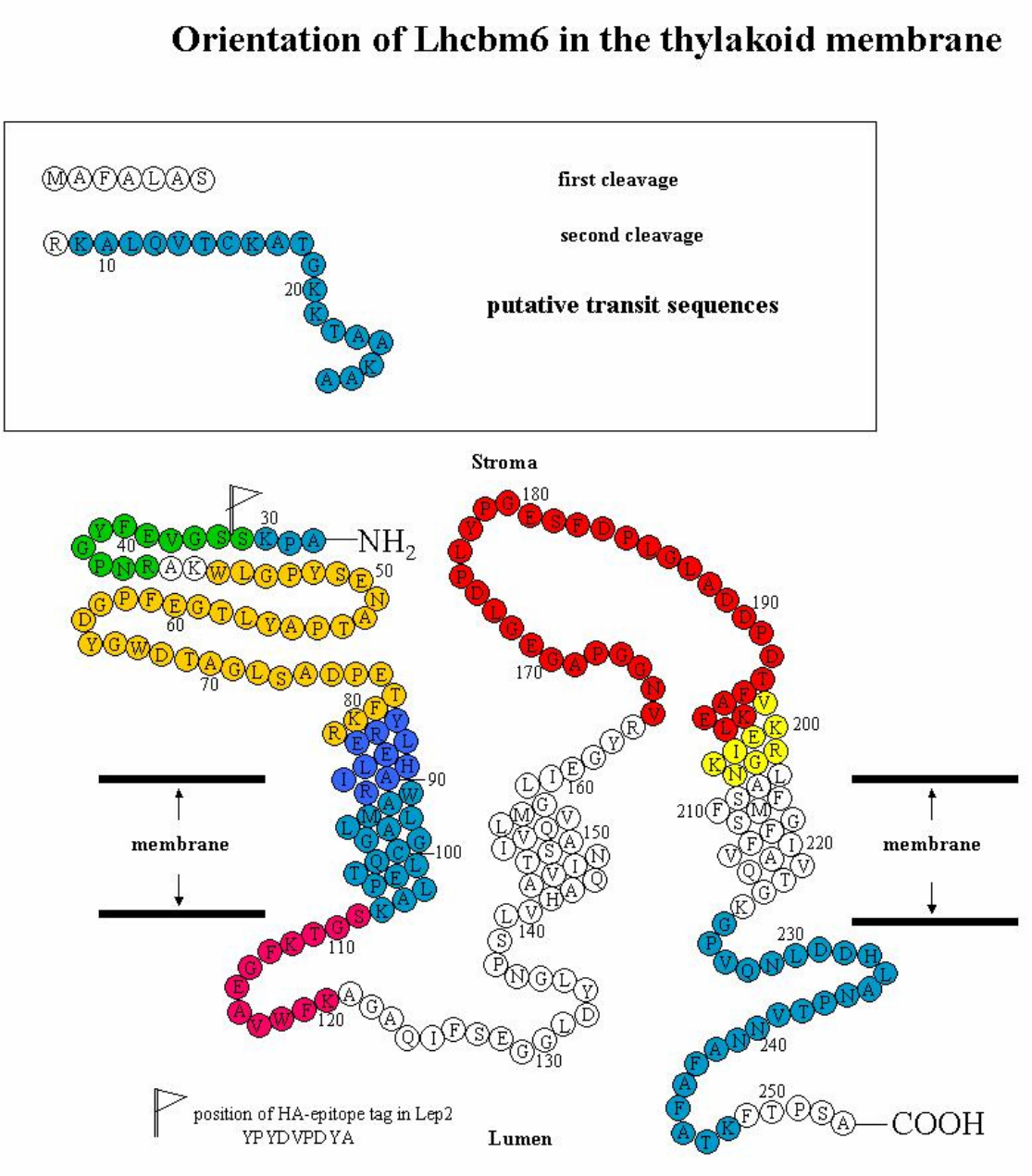


Fig. 25: Orientation of Lhcbm6 in the thylakoid membrane. The flag symbol indicates the position of the HA-epitope inserted in the lep2 mutant. Coloured amino acid residues refer to peptides identified by mass spectrometry (see Table 32). Residues coloured like ● refer to peptides that exclusively identify Lhcbm6.

1.5.) Integration of preprocessed Lhcbm3 and 6 in thylakoid membranes

Preprocessed Lhcbm6 assembles and integrates into the thylakoid membrane in a stable manner, since spot 22 can not be removed from thylakoid membranes by sodium carbonate extraction at pH 12 (data not shown). This suggests that the second processing event may occur at the thylakoid membrane. The fact that the tryptic peptide 9-16 of Lhcbm6 is found by mass spectrometric analysis of tryptically digested trimeric LHCII (Figure 25) suggests that preprocessed Lhcbm6 assembles into trimeric LHCII. These findings show that the two processing events which have been found by *in-vitro* studies of higher plant pre-Lhcbm6 (see above) also may occur in vivo in *C. reinhardtii.* However, the functional role of these alternative processing sites in the assembly or regulation of Lhcbm6 remains to be determined. For *C. reinhardtii* (Rüfenacht and Boschetti, 2000) described four distinct stromal processing proteases, indicating that processing of transit peptides is a rather complex process.

Thus, different stromal processing peptidases that process the transit sequence at different positions could be the cause for the different N-terminal protein products observed for Lhcbm3 or Lhcbm6. Such a processing event would remove the phosphorylation site at Thr3 in Lhcb1 of vascular plants and would thereby inhibit the ability of the respective Lhcb1 protein to migrate to the stromal membranes and participate in the process of state transitions.

1.6.) Phosphorylation of Lhcbm3

Our data indicate that Lhcbm3 is phosphorylated in *Chlamydomonas*. In the 2-DE map, Lhcbm3 is found in the upper row of Lhcbm protein spots (Fig. 8), corresponding to the position of protein P11, which is one of the major phosphorylated protein bands seen in SDS-PAGE analysis under state II conditions in *Chlamydomonas* (Wollman and Delepelaire, 1984). Interestingly, phosphorylation of the second Thr residue in the respective Lhcbm3 peptide [(K)GT*GKT*AAKQAPASSGIEFYGPNR] (Fig. 11) corresponds exactly to the phosphorylation of Thr3 in vascular plants (RKT*AAKAKQ...) (asterisks indicate phosphorylated residues) (Michel et al., 1991; Vener et al., 2001). The MS data identify two N termini of Lhcbm3 in which, due to N-terminal protein processing, both phosphorylation sites are removed. Therefore, it can be suggested that such N-terminal protein processing could be a regulatory event in the process of state transitions in *Chlamydomonas* as well as vascular plants.

1.7.) 2-dimensional phosphorylation map of thylakoid membranes

Phosphorylation profiles of *Chlamydomonas reinhardtii* thylakoid membranes have been obtained earlier (Fleischmann et al., 1999), but here for the first time a phosphorylation map of thylakoid membranes separated by 2-DE is presented (Fig. 12). In a onedimensional phosphorylation map (Fleischmann et al., 1999) the light harvesting proteins CP26, CP29 and LHCP11, 13 and 17 (Bassi

and Wollman, 1991) could be identified besides the PSII core protein D1 by autoradiography of *in vitro* γ-^{32}P-labeled thylakoid polypeptides. In our study CP26 and CP29 and Lhcbm3 and/or Lhcbm6 have also been found in their phosphorylated state. D1 has not been identified, either due to its low abundance in the preparation or due to the fact that it was not phosphorylated under the conditions favouring state transitions. For the phosphorylation pattern we see only one row of spots that correspond to Lhcbm proteins, which leads to the conclusion that the HA-tagged form cannot be phosphorylated or that the phosphorylation signal is only given by Lhcbm3 which is overlayed in the 2-DE pattern by Lhcbm6. Anyway, it is clear that Lhcbm3 is involved in the phosphorylation pattern, since Lhcbm6 is deficient in stm3, but it can not be ruled out that Lhcbm6 is also a target for the LHC kinase. The lower molecular weights on the phosphor screen could correspond to phosphorylated forms of Lhcbm1.

1.8.) 2-DE as a tool to identify the phenotype of mutants deficient in LHCII proteins

High resolution 2-DE and immunoblotting proved feasible for the Lhcbm depleted mutants npq5 and stm3 to demonstrate the deficiency of the respective deleted or non-expressed proteins (Fig. 15 and 16). Thus the 2-DE analysis of thylakoid membranes in combination with immunoblotting and mass spectrometry provides a valuable tool for functional proteomics, in order to elucidate the implication of specific LHCII proteins in photosynthesis and its controling and regulating mechanisms. By visualising the expression profile of LHCII proteins, in combination with the

phenotype of a mutant it becomes evident that single light harvesting polypeptides have a specific role in thylakoid membrane processes.

2.) Comparison of two Chl b-deficient mutant strains

Mutant strain S2 was created by insertional mutagenesis from the PsaF-deficient mutant 3bf and selected on HSM plates containing the antibioticum zeocin under high-light conditions. In Arabidopsis PSI-F is involved in plastocyanin docking to PSI but furthermore seems to be involved in energy transfer from LHCI to PSI and thylakoid organisation (Haldrup et al., 2000). Whereas the phenotypes of *Synechocystis PCC 6803* psaF-mutants suggested a dispensable, accessory function for this photosystem subunit in cyanobacteria (Chitnis et al., 1991; Xu et al., 1994), gene knockout studies in *Chlamydomonas reinhardtii* showed that electron transfer from plastocyanin to P700+ is drastically impaired in the absence of PsaF (Farah et al., 1995). In *Chlamydomonas reinhardtii* PsaF serves to bring plastocyanin into proper orientation for efficient electron transfer from PSII to PSI (Hippler et al., 1997; Hippler et al., 1998). Deletion of PsaF in *Chlamydomonas reinhardtii* thus leads to slowdown of electron transfer to PSI and to accumulation of transfer energy which ultimately provokes toxic effects on the photosystems by creating reactive oxygen species that lead to photoinhibition of PSII (Hippler et al., 2000). Western blotting experiments showed that the mutation in S2 did not restore PsaF-deficiency (Fig. 18), which leads to the assumption that high-light resistance could be caused by decrease in photon influx, probably through reduction of light harvesting antennae.

Furthermore chl analysis revealed deficiency in Chl b. Chl b-deficiency could be interpreted as either cause or consequence of redundancy in light harvesting proteins, for the mutant S2, whereas in the mutant cbs3 Chl b-deficiency caused by deletion of the gene encoding Chl a oxygenase (CAO) (Tanaka et al., 1998) is clearly the reason for decreased accumulation of light harvesting proteins.

2.1.) 77 K fluorescence

To get a first measure of the coupling of the LHCs and photosystems we analysed thylakoids of wildtype and mutants by 77K fluorescence emission spectra. Low temperature fluorescence emission peaks at 689 and 710 nm reflect the proportion of light energy absorbed by chlorophyll molecules which can not be directly coupled to light harvesting complexes or photosynthetic reaction centers (Wollman and Bennoun, 1982). The fluorescence peaks at 685 nm obtained by wildtype cells grown under normal conditions are characteristic for LHCII complexes coupled with PSII, whereas the measured peaks at 710 nm are characteristic for LHCI proteins coupled with PSI. The fluorescence curve for mutant strain S2 shows an undefined emission spectrum, which could be contributed both by uncoupling and reduction of LHCI and LHCII. In the mutant cbs3 there is a reduced peak at 705 nm which points both towards a reduction and decoupling of LHCI antennae. (Fig. 20) This is in contrast to an earlier study on this mutant using the kinetic- and spectrophotometric method (Melis and Anderson, 1983) and Western blotting to find that the LHCI antenna is only slightly reduced in comparison to wildtype (Polle et al., 2000).

2.2.) PAGE analyses

The observations from the low-temperature fluorescence measurements correlate with fractionation experiments of thylakoids which also proved that in the mutants cbs3 and S2 large portions of the LHCI proteins are disconnected from PSI. Whereas for wildtype after solubilisation, ultracentrifugation and fractionation of thylakoids 13 of 15 fractions are equally abundant in PSI and LHCI, respectively, most fractions in cbs3 and S2 contain different ratios of these protein complexes. (Fig. 21) In the mutant cbs3 this disconnection is also expressed in the absence of the PSI-subunit PsaK in PSI enriched fractions (Fig. 22). PsaK is a PSI subunit involved in the connection of PSI and LHCI. By cross-linking and reverse genetic studies PsaK is implicated in the connection of LHCI outer antenna to PSI (Jansson et al., 1996; Jensen et al., 2000). PSI-K has been suggested as a regulator of LHCI-PSI coupling. Under Fe-limiting conditions when accumulation of PSI is decreased because of its high Fe-content, PSI-K is supposed to change physical properties in order to disconnect LHCI from PSI thereby avoiding overexcitation of the antenna and photooxidative damage (Moseley et al., 2002a). Under Fe-deficient circumstances this process seems to be controlled by the Crd1 protein, a proposed Fe-containing enzyme involved in chl-biosynthesis, induced by iron-deficiency. Potentially a similar scenario could be evoked by a disturbance in chl-biosynthesis.

Uncoupling of LHCI from PSI was also nicely documented in the separation of native membrane complexes by Deriphat-PAGE and subsequent denaturing SDS-PAGE. Antibodies directed against specific LHCI- and PSI- subunits recognised PSI and LHCI in the same native band, whereas for the mutants these subunits were found in an additional band (cbs3) and exclusively in a different

band (S2). Surprisingly in cbs3 another PSI-fraction is found in a lower band. If this PSI-fraction remains LHCI proteins attached, it should be recognised by the anti-14.1-antibody, which is not the case, unless the recognition site of attached Lhca3 proteins cannot be detected in this complex due to modification, processing, or total detachment of the protein. (Fig. 23)

As the 2-DE experiments showed also assembly of LHCII is affected. Mass spectrometric measurements indicated that the reduced spot contains isoforms of Lhcbm1 and Lhcbm2/8. Since the other known Lhcbm1 spots are present, it might be obvious that Lhcbm2/8 is absent from this spot. On the other hand it is also possible that the specific isoform of Lhcbm1 or Lhcbm2/8 is not accumulated. (Fig. 19)

2.3.) Oxygen uptake assays

Measurement of oxygen uptake showed a higher value for light saturation in cbs3 and S2, being another pointer towards uncoupling of PSI and LHCI. The efficiency of light energy transfer in these mutants seems to be impaired so that maximum photosynthetic rates can occur at higher light intensities. This effect is more strongly observed in cbs3 compared to S2. (Fig. 24, Table 33) The severity of this effect in cbs3 could also be related to the strong redundance in PsaK, which could express a stronger degree of decoupling of LHCI from its photosystem. Thus Chl b-deficiency in cbs3 and decreased integration of Chl b molecules in LHCI seems to result in unstable association

of LHCI with PSI. Oxygen evolution measurements performed with cell suspensions of cbs3 by Polle and coworkers showed a slightly decreased photosynthetic rate for cbs3 when compared to wildtype, while the photon-use deficiency seemed to be decreased by half. In their study, however, saturation values of wildtype and cbs3 seemed to be similar (Polle et al., 2000).

2.4.) Pigment analysis

Looking at the data from the pigment analysis it is striking that the PsaF-deficient mutant 3bF shows an Nx/Vx ratio of 1.7 which is more than twice the wildtype ratio, whereas in the mutant S2 this value drops back to 0.32. In 3bF the observed changes are rather difficult to explain by phototoxic effects caused by the mutation, whereas for S2 higher total carotenoids levels and the decreased Nx/Vx ratio could give a hint to increased energy dissipation as a way to overcome photoinhibition. Violaxanthin is part of the xanthophyll cycle and although probably not a better quencher than neoxanthin, indispensible for npq, because violaxanthin can be reconverted into the efficient quenchers antheraxanthin and zeaxanthin (Niyogi et al., 1997; Polivka et al., 2002). β-carotene accumulation could point towards an improved preposition for xanthophyll synthesis and npq. Furthermore lutein is enriched over the β-branch derived carotenoids. (Table 34) The observed changes in pigment content could either be caused by redirection of biosynthetic pathways or by selective loss of specific pigments in the course of LHC reduction through LHC disassembly as suggested above. Although the data from this theses make a defect in LHC assembly and/or

biosynthesis more plausible to describe the phenotype of the mutant S2, it can not be excluded so far that a regulation mechanism with an impact on pigment biosynthesis is implied.

2.5.) Concluding remarks on the comparison of two Chl b-deficient strains

Whereas the Chl b-deficiency in cbs3 can confidently be accounted for by the absence of CAO, the factors causing Chl b-deficiency in S2 is still unclear, although there are some parallels in the phenotype between the two mutants. In both mutants a large portion of the LHCI antennae seems to be detached from the photosystem leading to reduction of photon-flux efficiency to the reaction centre. In cbs3 deficiency in the last step of Chl b-synthesis and the subsequential loss of Chl b could cause destabilisation of LHC proteins through proper membrane integration. It has been shown before that chl-incorporation is required for the assembly of functional LHC trimers (Hippler et al., 2000). The strong reduction of PsaK under Chl b-deficiency is surprising and points to its dependence on Chl b, which is unexpected but validated by the data. Such a mechanism is not implicated in the Chl b-deficiency phenotype of the mutant S2, since low amounts of Chl b might be left and as seen in mutant cbs3 knockout of Chl b-synthesis still allows binding of LHCI to PSI. Thus it is rather plausible that a factor involved in LHC biosynthesis and/or assembly is knocked out, and that Chl b is not incorporated due to lack of LHC proteins.

Summary

The photosynthetic machinery of the thylakoid membranes comprises about 100 protein complexes. Among them the light harvesting complex protein family forms a large subgroup, their large number bearing from a large number of nuclear-encoded genes, as well as posttranslational modifications. In this study 2-DE separation of thylakoid membranes from *Chlamydomonas reinhardtii* has been established as a tool for proteome mapping and functional analysis of light harvesting proteins, and dissection of mutant phenotypes affected in the expression and modification of thylakoid proteins.

A mutant screen has been applied to study the assembly of light harvesting proteins. In this screen a Chl b-deficient mutant was obtained that was characterised biochemically and physiologically. PAGE analyses and oxygen uptake assays implied that LHC proteins are reduced and detached from the photosystem. In the Chl b-deficient mutant cbs3 similar findings were obtained. Whereas effects on LHC proteins could be caused by Chl b-deficiency in cbs3, Chl b-deficiency in S2 is implied to be a consequence of LHC destabilisation, although the impact of regulation mechanisms in the context of pigment biosynthesis cannot be ruled out so far. Further pigment analysis looking at different growth conditions and measurement of npq could be valuable tools, especially if cbs3 is included in the analysis.

Unfortunately although highly elaborate state of the art skills were applied it was not possible to perform genetic crosses with the mutant S2 in order to correlate the introduction of the *ble*-gene with the phenotype of the mutant. After the mutant has been characterised biochemically in this study it would now be worthwhile to identify the affected DNA-segment by plasmid rescue.

From this study the following **theses** could be established:

1.) The separation of thylakoid membrane proteins isolated from *C. reinhardtii* by twodimensional gel electrophoresis demonstrated that transmembrane spanning proteins such as the LHCPs can be reliably separated by a high-resolution gel system. In addition the data show that this 2-DE system can be used for differential analysis of wildtype and mutant thylakoids.

2.) The separation of LHCPs from PSII and PSI by 2-DE enables differentiation between more than 30 LHCP spots. This number does not only reflect the number of genes encoding for this protein family, but also a high number of modification and processing events.

3.) The high level of heterogeneity of LHCPs in *Chlamydomonas reinhardtii* may have functional impact and assembly of a rather heterogeneous LHCI complex could be a way to modulate transfer of excitation energy to the PSI reaction center. Specific light harvesting proteins could have specific functions in the regulation and accomplishment of photosynthesis.

4.) Taking advantage of the sequence information provided by the *Chlamydomonas* genome project, which allowed to retrieve peptide sequences from a large *Chlamydomonas* EST database as well as from a genomic database by using the MS data, eight distinct Lhcb proteins could be predicted six of which were completely represented in the first *C. reinhardtii* genomic database assembly (version 1.0). For the other gene products, complete genes could not be reconstructed, but the genomic assembly did represent partial sequences.

5.) N-terminal protein processing of the *lhcbm3* and *lhcbm6* gene products could be identified. This finding, which was evident from MS data as well as from the Lhcbm6-HA tagging experiment,

demonstrates that two differentially N-terminally processed forms of Lhcbm3 and Lhcbm6 exist in thylakoid membranes.

6.) The fact that the tryptic peptide 9-16 of Lhcbm6 is found by mass spectrometric analysis of tryptically digested trimeric LHCII suggests that preprocessed Lhcbm6 assembles into trimeric LHCII.

7.) Mass spectrometry revealed a mass shift of 80 kDa for a tryptic peptide excised from a monomeric green gel band. This leads to the conclusion that the Lhcbm3 polypeptide is phosphorylated in thylakoid membranes of *Chlamydomonas reinhardtii*. By N-terminal protein processing, both candidate phosphorylation sites would be removed. Therefore, it can be suggested that such N-terminal protein processing could be a regulatory event in the process of state transitions in *Chlamydomonas reinhardtii*.

8.) In the Chl b-deficient mutant cbs3 Chl b-deficiency is correlated with a reorganisation of the LHCI-PSI complex, expressed for example in a strong downregulation of the PSI subunit PsaK and the detection of two PSI populations and by immunoblotting of thylakoids separated by 2-dimensional PAGE (Deriphat + SDS).

9.) For the mutant cbs3 as well as for the high-light resistant mutant S2 oxygen uptake assays show higher k_m and V_{max} values in comparison to wildtype, indicating a reduced photon-use efficiency for PSI, which is caused by a downregulation of LHCI proteins.

10.) In both Chl b-deficient mutants (S2 and cbs3) Chl b-deficiency is correlated with reduction of LHC proteins and disconnection of LHC proteins from the respective photosystem. Whereas in S2 Chl b-deficiency seems to be a consequence of LHC-downregulation, in cbs3 destabilisation and

disconnection of LHC proteins seems to be a result of Chl b-deficiency caused by the disruption of the Chl b-biosynthesis pathway.

Abkürzungen

β-C	β-carotene
β-DM	β-dodecylmaltoside
1-D	onedimensional
2-D	twodimensional
2-DE	twodimensional gel electrophoresis (IEF + SDS-PAGE)
Å	Ångström
Ala	alanine
A_o	primary acceptor of PSI
APS	ammoniumpersulfate
ATP	adenosine triphosphate
b_6f	cytochrome *b_6f* complex
BCA	bicinchoninic acid
BO_3H_3	boric acid
C. reinhardtii	*Chlamydomonas reinhardtii*
chl	chlorophyll
Chl a	chlorophyll a
Chl b	chlorophyll b
CID	collision induced decay
cm	centimeter
CNBr	cyanogen bromide
CO_2	carbon dioxide
$CoCl_2 \cdot 6H_2O$	cobaltdichloride
Cu	copper
$CuSO_4 \cdot 5H_2O$	coppersulfate
Cyt	cytochrome
Da	dalton
DCMU	(3-(3,4-dichlorphenyl)-1,1-dimethylurea),
DCPIP	Dichlorophenolindophenol
dd	double distilled

DEAE	Diethylaminoethyl
dest.	distilled
DIGE	2-dimensional difference gel electrophoresis
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
E	Einstein
E. coli	*Escherichia coli*
EDTA	ethylenediamine-tetraaceticacid 2Na 2 H_2O
ELIP	early light inducible protein
ER	endoplasmatic reticulum
ESI	electrospray ionisation
EST	expressed sequence tag
FAB	fast atom bombardment
Fd	soluble ferredoxin
FeS	bound iron sulfur acceptors of PSI
$FeSO_4 \cdot 7H_2O$	iron sulfate
FeS-Rieske	Rieske iron sulfur protein
GRAVY	grand average hydropathy score
h	hour
H	hydrogen
H_2O	water
H_2O_2	hydrogen peroxide
$H_3PO_4^-$	phosphoric acid
HA	hemagglutinine
HEPES	$C_8H_{18}N_2O_4S$
HLIP	high light inducible protein
HPLC	high performance liquid chromatography
HPO_3	hydrogen phosphate
HSM	high salt medium
I(E)P	isoelectric point
ICP	inductively coupled plasma
IEF	isoelectric focussing
IPG	immobilised pH gradient

K	Kelvin
K_2HPO_4	di-potassium hydrogenphosphat
kDa	kilodalton
KH_2PO_4	potassium di-hydrogenphosphate
l	liter
LC	liquid chromatography
LC-ESI	liquid chromatography electrospray ionisation
LED	light emitting diode
LHC	light harvesting complex
LHCI	light harvesting complex I
LHCII	light harvesting complex II
LHCP	light harvesting complex protein
Lu	lutein
Lys	lysine
MALDI	matrix assisted laser desorption ionisation
max.	maximum
$MgCl_2$	magnesium chloride
$MnCl_2 \cdot 4H_2O$	mangane dichloride
$Mo_7O_{24}(NH_4)_6 \cdot 4H_2O$	ammonium molybdate
MOPS	(3-[N-Morpholino]propanesulfonic acid)
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
NaCl	sodium chloride
NADP	nicotine adenine diphosphate
NaF	sodium fluoride
NaN_3	sodium azide
NH_4Cl	nitrogen chloride
NH_4HCO_3	ammonium hydrogencarbonate
npq	non-photochemical quenching
Nx	neoxanthin
O_2	oxygen
OD	optical density
P_{680}	reaction center chlorophyll of PSII

P_{700}	reduced form of the reaction center chlorophyll of PSI
P_{700}^{+}	oxidised form of the reaction center chlorophyll of PSI
PAGE	polyacrylamide gel electrophoresis
PBM	peribacteroid membrane
PC	plastocyanin
PQ	plastoquinone
PQH_2	reduced plastoquinone
PSI	photosystem I
PSII	photosystem II
PVDF	polyvinylidene fluoride
Q	plastoquinone
SDS	sodiumdodecylsulfate
Ser	serine
TAP	Trace, Acetic Acid, Phosphate
TEMED	N,N,N,N´- Tetra-methylethylenediamine
Thr	threonine
TOF	time of flight
Tris	tris-(hydroxy)-methyl amino ethane
V	Volt
Vx	violaxanthin
w/v	weight per volume
Z	zeaxanthin
$ZnSO_4 \cdot 7H_2O$	zincsulfate

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Fink A., Johanningmeier U., Hippler M.: Proteome analysis of *Chlamydomonas reinhardtii* thylakoid membranes to elucidate the modificational and functional diversity of photosynthesis-related gene products

- **Deutsche Botanikertagung 2002, Freiburg i. Br., 22. – 27. September 2002**

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